THE ANALYST

PROCEEDINGS OF THE SOCIETY FOR ANALYTICAL CHEMISTRY

DEATH

We record with regret the death of

Lewis Eynon.

NORTH OF ENGLAND SECTION

A Joint Meeting of the North of England Section of the Society and the North Lancashire Section of the Royal Institute of Chemistry was held at 7.30 p.m. on Friday, October 27th, 1961, at the College for Further Education, Torrisholme Road, Lancaster. The Chair was taken by the Chairman of the North Lancashire Section, Mr. T. E. V. Horsley, B.Sc., F.R.I.C.

The following paper was presented and discussed: "The Analysis of Edible Oils Contaminated with Synthetic Ester Lubricants," by G. B. Crump, B.Sc., A.R.I.C.

SCOTTISH SECTION

An Ordinary Meeting of the Section was held at 4 p.m. on Friday, October 20th, 1961, in the Chemistry Department, the University, Aberdeen. The Chair was taken by the Chairman of the Section, Mr. A. F. Williams, B.Sc., F.R.I.C.

The following papers were presented and discussed: "Stannous Acetate as a Primary Standard," by W. Moser, B.Sc., and W. B. Simpson, B.Sc.; "Some Seldom-remembered Aspects of Analytical Technique," by R. A. Chalmers, B.Sc., Ph.D.; "The Analytical Chemistry of Technetium and Rhenium," by R. J. Magee, M.Sc., Ph.D., A.R.I.C. (see summaries below).

The meeting was preceded at 2.15 p.m. by a tour of the Chemistry Department, Aberdeen University, by kind invitation of Professor G. M. Burnett.

Stannous Acetate as a Primary Standard

Mr. W. B. Simpson discussed the need for a pure, stable and readily soluble stannous tin compound. He gave a comparison of five different sources of stannous tin. Results showed that stannous acetate, purified by vacuum sublimation, was a suitable primary standard in tin analysis.

Some Seldom-remembered Aspects of Analytical Technique

Dr. R. A. Chalmers said that there were practically no channels of communication for discussion of minor sources of error, especially those that were conveniently forgotten. The information that existed was scattered over various texts, and attempts at publication in learned journals were usually blocked by refusal to accept "text-book material." Dr. Chalmers made this communication in an attempt to rectify this state of affairs and to draw attention to (or remind people of) such errors as those that occurred in weight calibration, instrument reading, dilution, desiccation, and so on.

The Analytical Chemistry of Technetium and Rhenium

Dr. R. J. Magee said that, along with manganese, technetium and rhenium constituted group VIIa of the periodic classification. Although rhenium had been discovered in 1925 by Noddack, Tacke and Berg, and technetium had first been obtained in 1937 by Perrier and Segré through neutron bombardment of a molybdenum target, the analytical chemistry of the two elements remained comparatively unknown until recent years. In this lecture Dr. Magee presented aspects of the analytical chemistry of these two elements, and in particular, work with reductants, such as bismuth amalgam. By this the 5-valent state of rhenium was established and the first satisfactory titrimetric method for the element developed. The use of bismuth amalgam and other reductants with technetium also produced the first non-radiochemical methods of detecting the element and led to new colorimetric methods for its determination.

WESTERN SECTION and MICROCHEMISTRY GROUP

A Joint Meeting of the Western Section and Microchemistry Group of the Society with the Mid-Southern Counties Section of the Royal Institute of Chemistry was held at 7.45 p.m. on Friday, October 20th, 1961, at the Red Lion Hotel, Salisbury, Wiltshire. The Chair was taken by the Chairman of the Microchemistry Group, Mr. C. Whalley, B.Sc., F.R.I.C.

The subject of the meeting was "Trace Analysis in Archaeology" and the following papers were presented and discussed: "Analytical Techniques in Archaeology and the Arts," by E. T. Hall, M.A., D.Phil.; "The Applications of Spectroscopy to the Study of Roman and Medieval Glazed Pottery," by J. W. G. Musty, A.R.I.C.

The meeting was preceded at 2.30 p.m. by a visit to Avebury Museum and Prehistoric Circle.

MIDLANDS SECTION

An Ordinary Meeting of the Section was held at 7 p.m. on Thursday, October 26th, 1961, at the Nottingham and District Technical College, Burton Street, Nottingham. The Chair was taken by the Chairman of the Section, Dr. H. C. Smith, M.Sc., F.R.I.C.

The following paper was presented and discussed: "Aquametry," by J. H. Thompson, B.Sc., Ph.D., A.R.I.C.

MICROCHEMISTRY GROUP

The thirty-first London Discussion Meeting of the Group was held at 6.30 p.m. on Wednesday, November 8th, 1961, at "The Feathers," Tudor Street, London, E.C.4. The Chair was taken by the Chairman of the Group, Mr. C. Whalley, B.Sc., F.R.I.C.

The subject for discussion was "Derivatography as a New Thermoanalytical Method," and it was opened by Professor L. Erdey, of the University of Budapest.

PHYSICAL METHODS GROUP

The seventy-ninth Ordinary Meeting of the Group was held at 6 p.m. on Tuesday, October 24th, 1961, in the Research Department Lecture Theatre, Boots Pure Drug Co. Ltd., Pennyfoot Street, Nottingham. The Chair was taken by the Chairman of the Group, Dr. G. W. C. Milner, F.R.I.C., A.Inst.P.

The subject of the meeting was "Determination of Particle Size," and the following papers were presented and discussed: "Recent Investigations into Sedimentation Methods of Particle Size Analysis," by B. H. Kaye, M.Sc.; "Automatic Scanning Instruments for Counting and Sizing Microscopic Particles," by W. H. Walton, B.Sc., F.Inst.P.; "The Use of an Electrolytic Resistivity Method (Coulter Counter) for Particle Size Analysis," by I. C. Edmundson, B.Pharm., M.P.S.

The meeting was preceded at 2.30 p.m. by a tour of the Standards Department Laboratories of Boots Pure Drug Co. Ltd., Beeston.

Obituary

HENRY VINCENT AIRD BRISCOE

HENRY VINCENT AIRD BRISCOE, who died on September 24th, 1961, his seventy-third birthday, was a pupil at the City of London School before entering the Royal College of Science where, in 1906, he was one of the last group of students to work in the chemistry laboratories in the "old College" in Exhibition Road, South Kensington. After graduation he was appointed to the academic staff, first as assistant to Professor Sir Edward Thorpe, then engaged in the revision of Thorpe's Dictionary of Applied Chemistry, and later as Demonstrator. He was a most conscientious teacher, and an indefatigable investigator in days when the facilities available were scanty—many a night was spent at the blowpipe or the workshop bench—and when the young lecturer (or indeed often the professor either) had no postgraduate student working under his guidance. His early researches consisted of accurate determinations of atomic weights, a subject which also engaged his attention in later years as Professor of Inorganic and Physical Chemistry at King's College (then Armstrong College), Newcastle (1921–32). Returning to Imperial College as Professor of Inorganic Chemistry he continued his investigations into the chemical behaviour of deuterium, fluorine, rhenium and germanium and their compounds. Work that he regarded as specially important related to the incidence of silicosis and involved microchemical analysis, which reflected his skill in conducting operations of high precision. Indeed he was one of the founders of the Microchemistry Group of the Society for Analytical Chemistry, of which he became a member in 1944 and in the same year was elected the first Chairman of the Group. By the same token he always took a particular interest in the analytical section of his department at Imperial College, where meanwhile he had become Director of the Laboratories for Inorganic and Physical Chemistry (1938–54); he regarded as of prime importance training in meticulous experimentation and precise measurement, and was instrumental in improving the facilities available there for micro, semi-micro and physical methods of analysis.

Professor Briscoe had held office in the Chemical Society, in the Society of Chemical Industry and in the Royal Institute of Chemistry, of which he had been a Vice-President for eleven years. Both during his active academic career and afterwards, when he was a guest of his old department, he kept in close touch with industrial aspects of his science, and was frequently called into consultation on a variety of topics, principally the chemistry of coke, paints, water supplies and dairy products. Throughout the 1939–45 War his services were freely offered to, and used by, Government agencies; in the years following the War he encouraged efforts to place the training of science laboratory technicians on a more satisfactory basis, and was himself Chairman of the Imperial College Technician Training Committee, of a joint Committee of the University of London Colleges having similar responsibilities, and of the National Joint Committee concerned with the recruitment, training and "deferment" of technicians.

Professor Briscoe will be remembered by all his associates not only as a distinguished chemist but also as a loyal colleague, full of human kindness as well as of worldly wisdom, and ever ready to extend a helping hand when the need arose.

A. A. ELDRIDGE

The Determination of Nucleic Acids in Biological Materials

A Review*

By W. C. HUTCHISON and H. N. MUNRO
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General recommendations for nucleic acid determination

WORK on the structure and function of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) continues unabated in intensity. Potter[1] has recently compared the growth of interest in the field of nucleic acids with the phases of growth of a bacterial culture. After preparation of the medium and inoculation with the basic discoveries, we are now in a phase of logarithmic growth of knowledge about the nucleic acids. A necessary part of this exploration of the function of the nucleic acids is the accurate measurement of the amounts of both types of nucleic acid in biological materials of all kinds. Further, the measurement of DNA has come to possess a wider interest, in that it can be used as a measure of cell number in tissues, and in consequence data on other cell constituents such as protein can conveniently be expressed in relation to DNA as a standard of reference,[2,3,4,5,6,7] a use foreshadowed by Berenblum, Chain and Heatley[8] in 1939.

The basic procedures currently in use for measuring the amounts of RNA and of DNA in biological materials—the methods of Schmidt and Thannhauser[9] and of Schneider[10]—

* Reprints of this paper will be available shortly. For details, please see p. 860.

were both enunciated in 1945, and there have since been several reviews of the field. The then available information was summarised in 1954 by Volkin and Cohn,[11] in 1955 by Markham[12] and in 1956 by Leslie,[13] and partial surveys have been made by Schmidt[14] in 1957 and by Webb and Levy[15] in 1958. Such is the rate of growth of knowledge in the nucleic acid field, however, that the time seems appropriate for a re-appraisal of methods of determining nucleic acid. An attempt is made here to cover in as complete a fashion as possible all published results with a bearing on the determination of nucleic acids.

As the title indicates, the methods reviewed here will be restricted to those applicable to the determination of nucleic acids in biological materials. Procedures used in purely chemical or other non-biological experiments will not be included; thus viscosimetric measurement is not dealt with. Also excluded from the survey are methods designed for measuring incorporation of radioactive precursors into RNA and DNA without reference to quantitative recovery of these nucleic acids.

In analysing the nucleic acid content of biological specimens, the initial step is to remove substances that would otherwise interfere with reactions used subsequently for determining the nucleic acids. This commonly involves washing the tissue briefly with acid in the cold, in order to remove acid-soluble small molecules (*e.g.*, free nucleotides), and this is usually succeeded by removal of the lipids and phospholipids with organic solvents. In some forms of nucleic acid determination, this sequence is reversed, the lipids being removed first. The end-product in either event is the acid-washed lipid-free tissue residue, on which the nucleic acids can now be determined.

The chemical constitution of the nucleic acids provides three main features that can be exploited for determining the amounts of RNA and DNA in the tissue residue. The nucleotide units from which RNA and DNA are constructed each consist of a purine or a pyrimidine base, a sugar (ribose in RNA, deoxyribose in DNA) and phosphoric acid. Consequently, the nucleic acids can be determined (*a*) by utilising the strong ultra-violet absorption of the bases, which will, however, not distinguish RNA from DNA, (*b*) by means of specific reactions for ribose (RNA) and deoxyribose (DNA) and (*c*) by determination of phosphorus, again a procedure that fails to distinguish RNA from DNA. Thus, if ultra-violet absorption or phosphorus determination is chosen as the basis of the analysis, it is evident that the two nucleic acids must first be separated before their concentrations can be measured independently. Such preliminary separation is the objective in the procedures of Schmidt and Thannhauser[9] and of Ogur and Rosen.[16] In the Schmidt - Thannhauser method, the different sensitivities of RNA and DNA to alkaline hydrolysis are utilised to separate them, and in the original form of this method the separated nucleic acids were then measured by phosphorus determination. The Ogur - Rosen procedure involves the extraction of RNA by prolonged exposure to cold perchloric acid and subsequent extraction of the DNA with hot perchloric acid; the ultra-violet absorption of each extract is a measure of the amount of RNA or DNA present. By contrast, the relatively specific reactions for ribose and deoxyribose can be used for measuring the amounts of RNA and DNA in solutions containing both nucleic acids; this is the basis of the Schneider[10] procedure, in which the nucleic acids are extracted by hot acid, the specific sugar reactions being then applied to this extract.

These three procedures, or modifications of them, are the main methods in current use for determining the RNA and DNA contents of biological materials; in this review each will be considered in turn. On occasion, other procedures have also been used, such as the quantitative isolation of uracil to represent the RNA in the sample and of thymine to represent its DNA content, and these will be dealt with later. Before considering the three main procedures and the less well known forms of determination, however, we shall first of all discuss in the preparation of biological materials for nucleic acid analysis those steps common to most methods of determination. Finally, at the end of the review an attempt will be made to provide the newcomer to this field with some guiding principles on which to base a choice of method appropriate to the material in which he wishes to measure the nucleic acid.

THE PREPARATION OF TISSUE SAMPLES FOR NUCLEIC ACID DETERMINATIONS

Precautions during removal of tissues—

The ubiquitous presence in tissues of enzymes capable of degrading nucleic acids to small molecules makes it essential to protect the nucleic acids while the tissue is being excised

and prepared for analysis. This has led most workers to transfer their samples as rapidly as possible to cold conditions, and it has been generally assumed that this is adequate to prevent significant enzymic degradation, although insufficient attention has been paid to verifying that this is so. Many workers homogenise the chilled tissue in water or saline in an ice-jacketed homogeniser and subsequently add ice-cold trichloroacetic acid (TCA) to precipitate the protein and extract the acid-soluble constituents, but other investigators have homogenised directly in TCA in order to minimise enzymic degradation. In theory this is commendable, but it must be remembered that TCA can attack the metal parts of a homogeniser, and with the Potter - Elvehjem type of homogeniser difficulty may be encountered due to the formation of the slightly sticky protein precipitate that tends to clog the space between pestle and tube.

Sometimes more strenuous precautions are taken to prevent enzymic degradation. Logan, Mannell and Rossiter[17] plunged their samples of nervous tissue into liquid nitrogen and then ground the tissue to a fine powder. In a review of procedures for nucleic acid determination suited to plant tissues, Markham[12] suggests that placing the tissue in boiling alcohol would be the most effective method of inactivating enzyme activity. Kovaćs[18] suggests the use of strong urea solutions for rapid solution of tissue culture cells. May and Grenell[19] studied factors affecting nucleic acid recoveries from different areas of rat brain. Removal and dissection of the brain took a maximum period of 15 minutes; unless the brain was dissected on a chilled block, nucleic acid recoveries were reduced by 20 to 37 per cent., according to the area of the brain analysed. In addition, these authors found that storage of brain tissue by deep-freezing led to a lower recovery of nucleic acids than was obtained from fresh tissues. This loss was confined to RNA and amounted to as much as 60 per cent. for the hypothalamus. On the other hand, Smith[20] observed no change in the RNA content of specimens of liver frozen for as much as 4 days. He found, however, that the temperature of preparation and homogenisation of the tissue was critical, dry ice giving 20 per cent. higher recoveries than cracked ice.

EXTRACTION OF ACID-SOLUBLE COMPOUNDS—

Brief extraction of the tissue with cold acid is used (*a*) to remove inorganic phosphate and organic phosphorus compounds of low molecular weight, as they might otherwise appear in the phosphorus from the nucleic acid fraction, (*b*) to remove sugars and polysaccharides that would affect sugar reactions for RNA and DNA and (*c*) to eliminate nucleotide coenzymes whose purine and pyrimidine components would augment the recoveries of these bases in the nucleic acid fractions. In selecting conditions for the extraction of these interfering compounds, the investigator must balance two opposing hazards: on the one hand, his conditions may be inadequate to extract these compounds completely; on the other hand, too vigorous extraction may result in some degradation of the nucleic acids. The latter possibility can take two forms. Injudicious use of acid can result in extraction at this stage of RNA along with the compounds of low molecular weight. Secondly, DNA may be attacked by the acid, causing loss of purine bases, through change to apurinic acid. The apurinic acid remains in the acid-insoluble precipitate, but, unlike the parent DNA, being sensitive to hydrolysis by alkali under the conditions of the Schmidt - Thannhauser procedure, then appears in the RNA fraction.

The extraction of acid-soluble compounds has usually followed the normal lines for obtaining the acid-soluble fraction of tissues. TCA is commonly used at a final concentration of 10 per cent., but as little as 5 per cent. may suffice. The tissues are homogenised in cold TCA and then subjected to at least one further wash with cold TCA, in order to achieve complete removal of acid-soluble material. It is important to maintain a low temperature during this extraction by making use of a refrigerated centrifuge, since not only does this minimise acid hydrolysis of the nucleic acid in the tissue, but it also favours formation of a granular precipitate of protein, which is effectively washed with TCA and lipid solvents. If the temperature is allowed to rise, the precipitate tends to be more or less gummy and is difficult to handle.

Studies of the completeness of the acid-extraction process have been almost exclusively confined to examination of phosphorus recoveries. McCarter and Steljes[21] subjected liver homogenates to four extractions with cold 10 per cent. TCA; their results show that the successive extractions accounted for 91, 4, 3 and 2 per cent. of the total acid-soluble phosphorus obtained. In a similar study of the acid-soluble phosphorus compounds extracted

from allantoic membranes by 5 per cent. TCA, Graham[22] has shown that three successive extractions provided 92, 7 and 1 per cent. of the total acid-soluble phosphorus obtained, and on studying extraction of liver samples with 10 per cent. TCA Moulé[23] observed that a fourth extraction removed only an additional 1 per cent. of phosphorus. The effect of repeated extraction of liver samples with 10 per cent. TCA has been more extensively investigated by Davidson, Frazer and Hutchison,[24] who obtained a small additional extraction of phosphorus compounds by increasing the number of extractions from 3 to 20, but consideration of the specific activity of this additional phosphorus fraction after ^{32}P injection led them to the conclusion that it was likely to have arisen from break-down of protein-bound phosphoric esters. This exhaustive procedure did not diminish recovery of phosphorus in either nucleic acid fraction subsequently isolated by the Schmidt - Thannhauser procedure. On the other hand, Lundin[25] observed some diminution in recovery of both RNA and DNA when washing with cold 7 per cent. TCA was increased from five to twenty times, and Daoust and Hooper[26] believe that extractions with cold TCA should be limited to four, so as to minimise break-down of acid-insoluble compounds. From all these investigations it seems clear that there is nothing to be gained by increasing the number of extractions of animal tissues with TCA beyond the conventional three or four times. On the other hand, yeast and bacteria contain polyphosphates that, if not extracted, will appear in the RNA fraction by the Schmidt - Thannhauser procedure. Removal of these substances appears to present some difficulties. Katchman and Fetty[27] extracted yeast with various concentrations of cold TCA for 1 hour and two subsequent 1-hour washes with 10 per cent. TCA. The concentration of TCA used in the initial extraction was varied between 5 and 40 per cent. without affecting the amount of orthophosphate recovered, but at the lower concentrations polyphosphate was inadequately extracted, whereas at the highest concentrations of TCA there was some break-down of nucleic acid. The authors finally selected 10 per cent. TCA as providing the best compromise. Finally, it must be remembered that acid-soluble tissue components other than phosphorus are potential sources of interference when the methods of nucleic acid determination do not depend on phosphorus measurements. Thus tissue glycogen can affect the determination of nucleic acids by sugar reactions. The experiments of Stetten, Katzen and Stetten[28] on extraction of glycogen from liver and muscle with 5 per cent. TCA show that removal of this polysaccharide is not measurably improved by increasing the number of extractions above three; there remains a fraction of tissue glycogen that defies extraction with TCA and is only solubilised by alkali.

Acid precipitants other than TCA have been used for removing compounds of low molecular weight. Cold perchloric acid (PCA) was introduced by two groups of investigators in 1950. Schneider, Hogeboom and Ross[29] extracted tissues once with 12 per cent. (2 N) PCA and then twice with 6 per cent. (1 N) PCA, whereas Ogur and Rosen[16] using plant tissues recommend two extractions with 1·2 per cent. (0·2 N) PCA. The latter authors state that no measurable amount of nucleic acid is lost by 3 hours' contact of the tissue with cold 0·2 N PCA, whereas essentially complete extraction of RNA from liver and plant tissues is obtained by exposure to 6 per cent. (1 N) PCA for 3 to 6 hours at 4° C. Hutchison, Downie and Munro[30] observed an appreciable loss of RNA from liver samples left in contact with cold 4 per cent. (0·7 N) PCA for 2 hours, and from embryonic tissues Paul[31] has recorded losses of RNA even with 1·2 per cent. (0·2 N) PCA used for a short period; with this tissue he has preferred to extract acid-soluble components with cold N sulphuric acid, to which RNA is stable. These observations indicate that there is an inherent danger of solubilising some of the RNA of the tissue if PCA is used as the extractant, notably when high concentrations are used.

A few authors have compared the efficiency of PCA and of TCA for removal of acid-soluble phosphorus compounds. Moulé[23] observed that four extractions with 5 per cent. (0·8 N) PCA at 4° C extracted the same amount of phosphorus from liver as did 10 per cent. TCA. Smillie and Krotkov,[32] in an extensive study of methods of nucleic acid determination on *Euglena gracilis*, subjected the organism to two extractions with 15 per cent. TCA, 10 per cent. (1·6 N) PCA or 2 per cent. (0·3 N) PCA. The two concentrations of PCA were less effective than the TCA in extracting phosphorus compounds, but the use of 15 per cent. TCA resulted, on the other hand, in a small loss of RNA. This was eventually circumvented by carrying out a preliminary extraction with methanol or other lipid solvents, after which acid-soluble phosphorus compounds could be completely extracted with 5 per cent. TCA or 5 per cent. (0·8 N) PCA.

The effect of allowing the temperature to rise during extraction with acid has been the subject of a study by Bonar and Duggan.[33] Samples of thymus DNA were treated with 5 per cent. TCA, 1·2 per cent. (0·2 N) PCA or 0·2 N hydrochloric acid for 40 minutes at room temperature. About 5 per cent. of the DNA phosphorus was dissolved during this treatment, but a more serious consequence was the production of apurinic acid by loss of purine bases, so that a large part of the DNA became susceptible to alkaline degradation under the conditions of the Schmidt - Thannhauser procedure for separating RNA from DNA. Consideration of this problem will be deferred until the Schmidt - Thannhauser procedure is discussed. Finally, some authors[16,31,34,35] have preferred to extract lipids before removing acid-soluble compounds. Although von Euler and Schmidt[36] observed that preliminary treatment with ethanol could render difficult the subsequent extraction of phosphorus compounds of low molecular weight, owing to their binding to protein, later workers have not encountered this difficulty. The work of Smillie and Krotkov,[32] cited above, indicates that preliminary treatment of the sample with methanol, acetone or ether permitted complete extraction of phosphorus compounds from Euglena with lower concentrations of acid than would otherwise have been needed. Graham[22] observed that the order of using 5 per cent. TCA and lipid solvents did not affect the recovery of acid-soluble phosphorus from allantoic membrane, and Fries[37] made a similar observation when studying the extraction of phosphorus compounds from leaves with cold 0·1 N hydrochloric acid.

From a consideration of all the available evidence on extraction of acid-soluble compounds from tissues, it seems that the use of TCA has much to commend it, three or four extractions with 10 per cent. TCA being adequate in most circumstances. The substitution of PCA for TCA should not be made lightly, unless there are compelling reasons for the change, such as the necessity to carry out ultra-violet measurements on the cold acid extract, for which PCA is much the better medium. If PCA is chosen, the drawbacks observed by some users should be borne in mind and steps taken to avoid them. The temperature of extraction must be rigorously controlled, but it seems a matter of indifference whether the extraction with acid comes before or after that with lipid solvents.

EXTRACTION OF PHOSPHOLIPIDS—

In many of the earliest procedures for nucleic acid determinations in tissues, the amount of nucleic acid was finally calculated from the phosphorus determination. This necessitated preliminary removal of phospholipids from the acid-extracted tissue residue by means of lipid solvents. With methods of nucleic acid analysis not dependent on phosphorus determination, it may not always be necessary to treat the tissue with lipid solvents. Thus Schneider[10] measured the nucleic acid content of tissues after treatment with cold acid and lipid solvents by extracting them with hot TCA and assaying the ribose and deoxyribose contents of the extract; he noted that omitting to use lipid solvents did not affect the values obtained for the RNA or DNA content of rat liver and rat brain. Similarly, Fleck and Munro[38] used ultra-violet absorption and ribose determinations in conjunction with the alkaline-digestion procedure of Schmidt and Thannhauser to determine the RNA content of rat liver and observed no effect on these values when lipid solvents were not used before the alkaline hydrolysis. There would thus seem to be good reason to omit the use of lipid solvents from nucleic acid analyses in which determination of phosphorus is not involved. Nevertheless, lipid solvents sometimes confer advantages other than removal of phospholipids. For plant tissues they often provide a convenient means of removing pigments. Moreover, TCA, which is strongly ultra-violet absorbing, is removed in the course of extraction with lipid solvents, and this may be advantageous if the nucleic acids are to be measured subsequently by their ultra-violet absorption. Removal of TCA at this stage is also a safeguard against its interference with the Ceriotti reaction for DNA.[39] Finally, recent evidence[40] shows the presence in yeast of nucleotide - amino acid complexes soluble in alcohol; whether failure to extract such compounds would contribute appreciable errors to measurement of nucleic acids in yeast or other biological material is not known.

Lipid solvents in considerable variety have been used for removing phospholipids from tissues.[41] It is not always easy to discern the principles that have guided each investigator's choice. The commonest method involves extracting the tissue residue after acid treatment with cold 95 per cent. ethanol and then boiling it in a (3 + 1) mixture of ethanol and ether.[10,21,23,25,42] Some authors then extract with ether[32,43,44] and occasionally with (1 + 1) methanol - chloroform[9,26,45,46] or methanol - ether.[47] Schneider and Klug[48] concluded that

two extractions with ethanol were sufficient to remove the phosphorus-containing lipids, but this is in contradiction to the known solubilities of the various phospholipids. The only solvent common to the three main groups of phospholipids is chloroform. The lecithins are extractable with cold ethanol and with ether, and the cephalins with ether; the sphingomyelins, on the other hand, are insoluble in cold ethanol and ether, but extractable with hot ethanol.[49,50] Consideration of these facts explains why McCarter and Steljes[41] found that, after extracting rat liver twice with ethanol, a considerable amount of phosphorus could still be extracted by one treatment with hot ethanol - ether, though little more phosphorus was obtained by further extraction with this solvent. Smillie and Krotkov[32] used boiling ethanol as their initial lipid extractant for Euglena cells, and this may account for their observation that subsequent extractions with hot ethanol - ether and with hot ether did not add significantly to the amount of phosphorus extracted. Daoust and Hooper[26] observed that ethanol and ethanol - ether extracted almost all the phospholipid material from liver, but that further traces were removed by subsequent extraction with methanol - chloroform and ether.

It may be concluded from the available evidence that two extractions with ethanol (removing TCA and lecithins) and one with hot ethanol - ether (removing cephalins and sphingomyelins) will prove adequate in most circumstances. However, it is often inconvenient to use hot solvents, and introduction of an extraction with cold chloroform will avoid this necessity. Since the specific gravity of chloroform is such that subsequent spinning in a centrifuge of the tissue residue is difficult, it is usual to employ a mixture of chloroform and ethanol, commonly in the proportions of 1 to 3.[8] A final wash with ether may be included if a residual dry powder is wanted. This dry powder is usually prepared by allowing the ether to evaporate at room temperature, but it is desirable to stir the material frequently to prevent formation of lumps, finely divided residue being more readily handled in subsequent treatment with acid or alkali. The standard method of lipid extraction used by us consists in treating the acid-extracted tissue with ethanol, ethanol - chloroform (3 + 1) twice, ethanol - ether (3 + 1) and ether.

Some authors have begun the lipid extraction with acetone.[24] This removes TCA, water and pigments, but the phospholipids in general are not soluble in acetone. Holloway and Ripley[51] found that the use of acetone overcame their difficulty in determining the nucleic acids of reticulocytes, whereas the use of ethanol immediately after TCA extraction caused formation of a gel.

A few authors have described adverse effects of lipid-solvent treatment on the recoveries of nucleic acids. Venkataraman and Lowe[52] and Venkataraman[53] have made the rather startling claim that about 30 per cent. of rat liver RNA is lost when the acid-wet tissue after extraction with cold 5 per cent. TCA is put in contact with cold 95 per cent. ethanol. Hutchison, Downie and Munro[30] have been unable to substantiate these observations. It may be noted that Schneider,[10] in the original description of his procedure for nucleic acid determination, prescribes initial extraction of the tissue with cold 10 per cent. TCA and then treatment with approximately 80 per cent. ethanol and afterwards other lipid solvents, but comments that omission of the lipid-solvent treatment does not influence the amount of RNA subsequently extracted from the tissue. This observation has been confirmed by Greenbaum and Slater[54] on mammary gland tissue, and Cooper and Loring,[55] with tobacco-leaf chloroplasts, have also observed that omission of preliminary treatment with TCA and lipid solvents (ethanol, ethanol - ether and ether) does not influence the recovery of RNA. These various observations lead to the conclusion that loss of RNA in cold acid-wet lipid solvents does not normally occur.

A different type of hazard arising from the use of lipid solvents has been described by Marko and Butler.[56] They observed that, after extraction of the tissue with cold TCA, two washes with cold ethanol were insufficient to remove all the acid and that enough remained to cause degradation of DNA to apurinic acid when the tissue was then heated in ethanol - ether. They were able to prevent this by saturating the first ethanol wash with sodium acetate, thus reducing the acidity. Lundin[25] has confirmed from analyses made on rat liver that degradation of DNA can occur if the degree of acidity is not controlled during lipid extraction and that Marko and Butler's modification effectively eliminates this action. The use of sodium acetate at this stage would seem a simple precaution to take in all forms of lipid-extraction procedure in which one of the solvents is heated or even kept for any length of time at room temperature. We ourselves[30] have encountered a variant of this difficulty

when samples of rat liver were extracted with cold PCA and then with lipid solvents and finally stored as dry powder at room temperature while awaiting analysis. During storage there was a gradual fall in DNA content, as measured by the Schmidt - Thannhauser method, and examination showed that the powders were still distinctly acid in reaction.

METHODS OF DETERMINING NUCLEIC ACIDS IN THE TISSUE RESIDUE

1. The procedure of Schmidt and Thannhauser

Various procedures are available for determining nucleic acids left in the tissue residue after treatment with cold acid and lipid solvents. Schmidt and Thannhauser's[9] method for this is outlined in the flow diagram (see Fig. 1), which shows that, after extraction of the tissue with acid and lipid solvents, the residue is incubated with alkali. This degrades RNA to acid-soluble fragments, leaving the DNA in an acid-insoluble form. Addition of acid at the end of digestion thus results in an acid-soluble fraction containing the RNA degradation products and a precipitate containing the DNA.

In the procedure originally described, the tissue residue is digested in N potassium hydroxide at 37° C for at least 15 hours, but many variations in the conditions for alkaline hydrolysis have been used, and their merits require evaluation. In the original method, moreover, phosphorus determination is used to measure the amounts of nucleic acid present in each fraction of the acidified digest. Measurement of the pentose and purine contents of the RNA fraction led Schmidt and Thannhauser to conclude that the results obtained by phosphorus determination gave a reliable measure of the RNA present. Nevertheless, a considerable body of evidence has since accumulated to show that, at least for the RNA fraction, phosphorus determination may involve considerable error from the presence of contaminating compounds. In consequence, other procedures for determining RNA and DNA in the isolated fractions of the alkaline digest have been proposed and require critical consideration.

Each of the features of the Schmidt - Thannhauser procedure—alkaline digestion, determination of RNA in the acid-soluble fraction and determination of DNA in the precipitate —will now be considered in more detail.

The use of alkaline hydrolysis to separate RNA from DNA—

It has been known for more than half a century that RNA undergoes degradation on exposure to alkali. In 1922, the foundations of the modern analytical use of alkaline hydrolysis were laid by Steudel and Peiser,[57] who demonstrated that yeast RNA is hydrolysed by sodium hydroxide acting at 15° to 17° C over a period of 24 hours, whereas thymus DNA is not attacked under these conditions. As shown in Fig. 2, the ease of hydrolysis of RNA (I) by alkali is due to the proximity of the hydroxyl group on the C_2 of ribose to the phosphoric acid of the 3′,5′-phosphodiester linkage, which permits formation under alkaline conditions

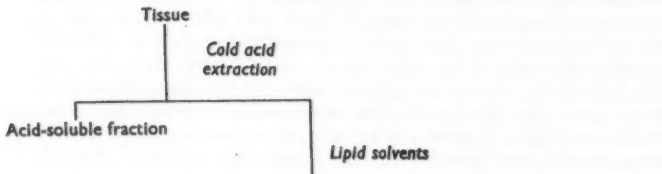

Fig. 1. The Schmidt - Thannhauser procedure for separation of RNA and DNA in tissues. (Reproduced from Davidson, J. N., "The Biochemistry of the Nucleic Acids," Fourth Edition, Methuen & Co., Ltd., London)

of a cyclic phosphotriester (II) involving the C_2 and C_3 atoms of the ribose. The triester then undergoes hydrolysis at the C_5 linkage, leading to rupture of the RNA nucleotide chain (III) and as final products the 2'- and 3'-mononucleotides (IV and V). The absence of a hydroxyl group on the C_2 atom of deoxyribose precludes the formation of a cyclic triester from DNA and thus accounts for the resistance of DNA to alkaline hydrolysis. This explanation of the differing actions of alkali on RNA and on DNA was first offered by Fonó[58] and has been amply supported by Brown and Todd.[59] Jordan[60] provides a useful recent summary of the chemical evidence about this series of reactions.

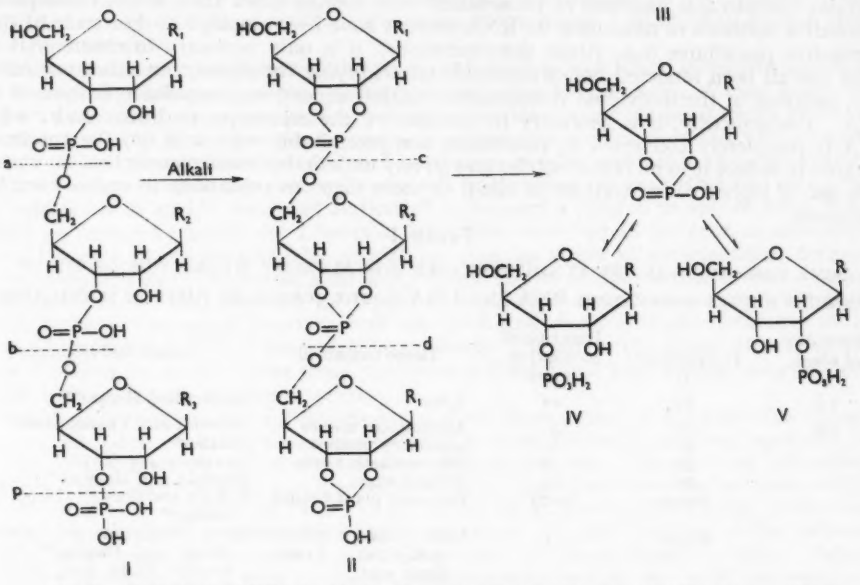

Fig. 2. Hydrolysis of RNA by alkali; R represents a purine or pyrimidine base. (Reproduced from Davidson, J. N., "The Biochemistry of the Nucleic Acids," Fourth Edition, Methuen & Co. Ltd., London)

The difference between the alkali labilities of RNA and DNA forms the basis of the Schmidt - Thannhauser procedure for determining the nucleic acids; in it the tissue residue, after treatment with cold acid and lipid solvents, is dissolved and incubated in alkali. The DNA is subsequently precipitated by acidification of the digest, whereas the RNA is not, because it has been hydrolysed by the alkali to simpler substances no longer precipitable by acid. It has been generally assumed that the products of RNA hydrolysis are entirely mononucleotides, but recent evidence shows that break-down is often less complete, a fact that can affect some of the procedures used in determining the amount of RNA obtained. This makes it necessary to consider in some detail the action of alkali on RNA.

Conditions for alkaline hydrolysis of RNA—Various alkalis have been used for hydrolysing RNA. The earliest studies on nucleic acid structure were carried out with ammonia, usually at high temperatures produced by autoclaving the sample.[61,62,63,64] A few authors have also used sodium carbonate,[65,66] barium carbonate[67] or the hydroxides of lead, cadmium, zinc, bismuth or aluminium,[68] the latter series of compounds acting by a mechanism not dependent on alkaline conditions. For analytical purposes, however, only sodium and potassium hydroxides have been employed as hydrolysing agents. In their degradative action on RNA, sodium and potassium hydroxides do not appear to differ in effectiveness, but potassium hydroxide possesses the advantage that most of the K^+ can be conveniently removed as the sparingly soluble potassium perchlorate by acidifying with perchloric acid at the end of alkaline digestion.

In their original description of the use of alkali for the quantitative separation of RNA from DNA, Schmidt and Thannhauser[9] prescribe the use of N potassium hydroxide at 37° C for at least 15 hours to render all the RNA non-precipitable with acid. The RNA content of the specimen is indicated by the amount of phosphorus not precipitated on acidification of the digest. Table I shows that many subsequent users of the Schmidt - Thannhauser principle have altered the conditions of alkaline hydrolysis; the strength of alkali has been reduced to as little as 0·05 N and the duration of digestion to as short a period as 30 minutes, and the temperature of incubation has been varied within a range extending from 5° to 100° C. Further, it has been found that the acid-soluble portion of the digest from many tissues contains considerable amounts of phosphorus from sources other than RNA; consequently alternative methods of measuring its RNA content have been employed. For some of these alternative procedures (*e.g.*, ribose determinations), it is only necessary to ensure that the RNA has all been rendered non-precipitable on acidifying the digest, but other procedures (*e.g.*, isolation of the individual ribomononucleotides) depend on complete hydrolysis of the RNA. Consequently, it is necessary to consider (*a*) the minimum conditions under which RNA is completely converted to substances non-precipitable with acid, (*b*) the conditions required to reduce it to mononucleotides and (*c*) any undesirable consequences that may result from use of higher concentrations of alkali or more vigorous conditions to ensure complete hydrolysis.

TABLE I

SOME VARIATIONS IN THE CONDITIONS USED FOR ALKALINE HYDROLYSIS OF TISSUE RESIDUES BEFORE SEPARATING RNA AND DNA IN THE COURSE OF THEIR DETERMINATION

Normality of alkali	Temperature, °C	Duration of incubation, hours	Tissue examined	Author and reference
1·5	37	48	Liver	Daoust and Hooper[36]
1·0	37	15+	Mammalian tissues	Schmidt and Thannhauser[9]
	37	4	Mammary tumour	Drasher[69]
	38	2	Haemopoietic tissue	Lowrance and Carter[70]
	30	18	Arbacia eggs	Marshak and Marshak[71]
	Room	18–24	Bacteria; plant tissues	Iverson and Giese[34]; Cooper and Loring[65]
	Room	1	Liver; kidney; spleen; epidermis; yeast; plant root	Scott, Fraccastoro and Taft[72,73]; Bonar and Duggan[33]; Griesemer[74]; Fleck and Munro[38]; Heyes[75]
	5	18	Liver nuclei	Olmsted and Villee[76]
0·5	38	18	Liver	Annau[77]
	37	24	Mammalian tissues	Montreuil and Boulanger[78]
0·4	100	1	Tissue culture cells	McIntire and Sproull[79]
0·3	37	1	Liver	Fleck and Munro[38]
	30	18	Fungi	Minagawa, Wagner and Strauss[80]
0·25	37	24	Liver	Kosterlitz[43]
0·1	37	18	Salivary gland; tumour cells	Rabinovitch, Rothschild and Junqueira[81]; Pileri and Ledoux[82]
0·05	80	0·5	Cell nuclei	Nash[83]
	35	4	Bacteria	Bernlohr and Webster[84]

The minimum concentration of alkali that will initiate degradation of RNA was investigated by Zittle,[85,86] who incubated samples of purified RNA with different concentrations of sodium hydroxide at 20° to 25° C for several days and observed the changes in the precipitability of RNA by a solution of uranyl chloride in TCA and also the liberation of acid groups from hydrolysis of the RNA. There was no evidence of degradation in samples held at pH 10·6 (equivalent to a final concentration of 0·0005 N alkali), but at pH levels above 11·8 (0·006 N alkali) reduction in precipitability and liberation of acid indicated that hydrolysis was taking place. Incubation at higher temperatures reduced the critical pH at which hydrolysis occurred. The stability of RNA in dilute alkali (pH 10·5 at room temperature) was confirmed by Brown, Fried and Todd.[87] This resistance to weak alkali has been used in the technique for removal of amino acids from soluble RNA.[88] After exposure of this RNA

to pH 10 for a period of 1 hour at 37° C, the soluble RNA still retains its biological activity of accepting labelled amino acids.[89] Although Zittle's results show that degradation occurs when RNA is exposed for a long enough time to concentrations of alkali above 0·006 N, it must be remembered that acidic groups are released as digestion proceeds, so that during incubation the pH may fall below the critical level for hydrolysis. Moreover, tissue samples contain proteins as well as nucleic acids, and the former will to some extent reduce the initial pH of the digest by buffering some of the alkali. For these reasons, it is necessary to use a considerably higher concentration of alkali than 0·006 N sodium hydroxide to ensure that the initial pH will be adequate in presence of tissue proteins and that hydrolysis will continue throughout incubation. The effective concentration will depend on the amount of tissue suspended in a given volume of alkali; a common proportion is 1 ml of alkali to each 100 mg of original tissue.[9]

Several authors have examined the products formed from RNA at different times during the course of alkaline digestion. Although Jones and Perkins[90] found that yeast RNA had to be exposed to 1 per cent. (0·25 N) sodium hydroxide at room temperature for 3 to 4 hours before it ceased to give a precipitate with acid, subsequent studies show that this estimate is unduly pessimistic. Within 15 minutes of incubation in 0·3 N alkali (pH 13·5), purified yeast RNA is rendered almost completely dialysable[91] and purified rat-liver RNA can no longer be precipitated with acid.[38] Lane and Butler[92,93] hydrolysed a purified sample of yeast RNA in 0·86 N potassium hydroxide for 2 hours at 26° C and recovered 48 per cent. as mononucleotides, 31 per cent. as dinucleotides and 21 per cent. as higher oligonucleotides; nothing in the hydrolysate was precipitable with acid. With digests of lipid-extracted tissue residues, it has been shown that exposure for 1 hour to N alkali at room temperature[33,73,74] or 0·3 N alkali at 37° C[38] is enough to extract all the RNA and destroy its precipitability by acid. It can be concluded that, if the objective is merely to separate the tissue RNA from DNA, then 1 hour of incubation under these conditions will suffice, at least for the tissues examined (liver, kidney, spleen, epidermis, yeast).

Although these studies indicate that the initial degradation of RNA by alkali proceeds rapidly, complete resolution to mononucleotides is more difficult to achieve, especially when the material submitted to digestion consists of tissue residues. Chargaff and his colleagues subjected purified yeast RNA and liver RNA[94] and the lipid-free tissue residue from sea-urchin eggs[95] to digestion at pH 13 to 14 (0·1 to 1 N alkali) for 12 hours at 30° C; they claim to have obtained in the RNA fraction of the digest essentially complete recovery of the ultra-violet absorbing material in the form of mononucleotides. Although Marrian, Spicer, Balis and Brown[96] also observed a high recovery of ribomononucleotides (86 to 99 per cent.) after digestion of purified yeast RNA in 0·3 N potassium hydroxide for 18 hours at 37° C, a much less satisfactory recovery was obtained when the material digested consisted of fat-free tissue residues. This is in agreement with the low recoveries of adenylic acid from de-fatted yeast specimens observed under similar conditions of alkaline digestion by Kerr, Seraidarian and Brown.[97] An examination of the recovery of mononucleotides by column chromatography after digestion of the lipid-free residue from rat liver at 37° C in different concentrations of alkali has been reported by Lamirande, Allard and Cantero.[98] After incubation in 0·3 N potassium hydroxide for a period of 24 hours, only 70 per cent. of the initial ultra-violet absorbing material of the original hydrolysate was recovered as mononucleotides. After incubation in N alkali for 42 hours the yield was 90 per cent.; with 1·5 N potassium hydroxide the recovery rose to 98 per cent. In calculating recoveries, these authors do not indicate how they corrected for ultra-violet absorption due to protein and to DNA in the material obtained at the end of alkaline digestion.

Although these earlier studies on mononucleotide recoveries from alkaline digests reveal a somewhat contradictory picture, the occurrence of a fraction in RNA resistant to alkaline hydrolysis is now well authenticated. Several authors[92,93,99,100,101,102,103] report the presence of material other than mononucleotides in hydrolysates obtained from purified samples of RNA subjected to digestion in N alkali at room temperature or at 37° C for periods of 18 to 24 hours. This alkali-resistant oligonucleotide material does not usually exceed 3 to 5 per cent. of the total nucleotides present; a claim by Potter and Dounce[101] that this fraction can attain 38 per cent. of the RNA in pancreas has not been confirmed.[93] More recently, Finamore and Volkin[103] have observed 10 and 20 per cent. of alkali-resistant oligonucleotide material in two species of RNA separated from amphibian eggs. The extensive researches of Lane and Butler[93] show that the oligonucleotide fraction of RNA is composed chiefly of

purine dinucleotides, notably diadenylic acid, which is only slowly hydrolysed by alkali. Samples of RNA prepared from different sources were found to vary in their yield of dinucleotides, a specially high yield being obtained from digests of a synthetic polymer of adenylic acid.[92] Most of this dinucleotide material is eventually hydrolysed to its mononucleotide constituents when the action of alkali is sufficiently prolonged, but the group also includes a number of dinucleotides permanently resistant to alkaline degradation.[93] This is apparently due to the presence of a sugar other than D-ribose, which prevents the formation of the cyclic triester essential to alkaline degradation of RNA and has been tentatively identified as 2′- or 3′-*O*-methylribose.[104]

From the available evidence, it can be concluded that determination of RNA in biological specimens by summation of its constituent mononucleotides may encounter the technical difficulty of incomplete hydrolysis. This can occur because (*a*) the effective alkalinity is reduced owing to the presence of protein in tissue residues submitted to digestion and (*b*) because significant amounts of alkali-resistant linkages are present in the RNA. From the results of Lane and Butler,[93] quoted above, it is apparent that the proportion of these linkages can vary from one species of RNA to another. Most of these oligonucleotides eventually undergo hydrolysis to mononucleotides,[93] and it would be logical to prescribe more vigorous alkaline conditions in order to achieve complete hydrolysis of the RNA. We must therefore consider whether an increase in the concentration of alkali or in the temperature of incubation, or both, and in the duration of incubation will lead to any undesirable effects.

One factor setting an upper limit to the conditions of alkaline digestion is the level at which DNA begins to degrade to acid-soluble products; evidence about this will be considered later. A second factor affecting some assay procedures is the deamination of cytidylic acid to uridylic acid by alkali. Early investigators in the field of nucleic acid chemistry were well aware of this possibility[66,105]; the reaction occurs with greater facility than deamination of free cytosine by alkali.[106] Marrian, Spicer, Balis and Brown[96] and Davidson and Smellie[107] observed significant deamination (10 to 30 per cent.) when pure solutions of cytidylic acid were incubated at 37° C in N alkali for 12 to 18 hours, but none after incubation in 0·3 N alkali at 37° C or in N alkali at room temperature. This last observation was confirmed by Loring, Bortner, Levy and Hammell[108] and by Hall and Allen.[109] Kleinschmidt and Manthey[35] observed 2·4 per cent. deamination after exposing cytidylic acid to 0·3 N potassium hydroxide for 18 hours at 37° C. Fleck and Munro[38] also observed deamination of cytidylic acid during digestion of purified rat liver RNA for 24 hours in N potassium hydroxide, but not in 0·3 N potassium hydroxide. On the other hand, Lamirande, Allard and Cantero[98] did not observe reduced recoveries of cytidylic acid when tissue residues were digested in concentrations of alkali as high as 1·5 N for 42 hours at 37° C, and Mandel, Weill and Ledig[110] also failed to note any difference in cytidylic acid recovery from alkaline digests of tissue residues over a 15-hour period at 37° C with either N or 0·33 N sodium hydroxide. The differing observations made with pure nucleotide solutions or purified RNA on the one hand and with digests of tissue residues on the other may represent a difference in effective concentration of alkali; the tissue residue will reduce the pH of the alkali used through the buffering action of its protein content and also by release of acidic groups during digestion.

The final factor that may deter the investigator from increasing the concentration of alkali or temperature of incubation is the concomitant release of protein in acid-soluble form from the tissue residue. This has long been recognised as an accompaniment of alkaline digestion. Fleck and Munro[38] have demonstrated that the amount of protein solubilised by alkaline digestion increases progressively with the period of incubation and to a smaller extent when the concentration of alkali is raised from 0·3 to 1 N at 37° C. Since these authors observed that RNA is fully extracted from liver in a form not precipitable with acid after exposure to 0·3 N potassium hydroxide for 1 hour, further prolongation of incubation merely releases more protein without improving RNA recoveries. The additional protein has the disadvantage of absorbing ultra-violet light and provides in consequence a source of error in measuring ribonucleotides by their ultra-violet absorption. On the other hand, a period of 1 hour in alkali is insufficient to convert the RNA to mononucleotides; if this is also desired, a two-stage digestion of the tissue residue can be employed. Thus Olmsted and Villee[76] extracted the tissue with N potassium hydroxide overnight at 5° C, precipitated the protein and DNA by acidification and subsequently continued digestion of the acid-soluble fraction in N alkali at room temperature. A modification of this procedure could be considered for

reducing to negligible proportions the extraction of protein by alkali and yet retaining its digestive action to the mononucleotides.

From a consideration of all the available evidence about the action of alkali on RNA, certain conclusions seem reasonably well founded.

(*a*) The minimum concentration of alkali needed for the extraction and degradation of RNA in tissues cannot be accurately assessed, on account of the buffering action of the tissue proteins. In practice, incubation in 0·3 N alkali at 37° C has proved adequate, and there seems to be no advantage in using lower concentrations.

(*b*) With 0·3 N alkali at 37° C or N alkali at room temperature all the RNA in a tissue can be extracted in 1 hour in a form no longer precipitable by acid. Although this does not degrade the RNA quantitatively to mononucleotides, the rendering of RNA non-precipitable with acid suffices for many analytical purposes.

(*c*) Considerably more prolonged incubation in alkali is needed to reduce the RNA completely to mononucleotides, which is necessary if the RNA is to be estimated as mononucleotides.

(*d*) Extraction of protein and possibly also deamination of cytidylic acid (both of which influence the ultra-violet measurement of RNA) can be unwelcome accompaniments of more prolonged incubation. However, complete degradation to mononucleotides can, when desired, still be attained without the need to extract much protein if a two-stage alkaline digestion is used.

(*e*) The efficiency of many of the variations in alkaline digestion shown in Table I has not been critically investigated. A period of 1 hour in 0·3 N alkali at 37° C or N alkali at room temperature is recommended as suitable.

Resistance of DNA to alkaline hydrolysis—The success of the original Schmidt - Thannhauser[9] procedure for separating RNA from DNA depends on the resistance of DNA to N alkali during 15 hours of incubation at 37° C. Since the original conditions of Schmidt and Thannhauser have frequently been modified, it is desirable to know the limits of resistance of DNA to alkali. Several studies indicate the conditions under which alkali can attack DNA at temperatures greater than 37° C. Bredereck and Müller[111] claimed to have degraded DNA to acid-soluble oligonucleotides by incubation in 0·1 N sodium hydroxide at 60° C, but Hurst[112] points out that their procedure for preparing DNA would itself reduce the DNA to oligonucleotides. Nash[83] found that thymus DNA resisted treatment with 0·05 N sodium hydroxide at 80° C for 30 minutes. A number of studies on the degradation of calf thymus DNA by incubation in sodium hydroxide at 100° C shows that the concentration of alkali is critical. Heating for 1 to 2 hours in concentrations of sodium hydroxide up to 0·6 N did not cause the release of more than 8 per cent. of the DNA phosphorus in acid-soluble form,[112,113,114] whereas incubation in N sodium hydroxide at 100° C resulted in the production of acid-soluble products accounting for 30[114] to 75 per cent.[112] of the DNA preparation after 1 hour and complete solubilisation after 4 to 8 hours at this temperature. Since the Schmidt - Thannhauser procedure in its original form prescribes incubation in N alkali at 37° C, it would be desirable to know the critical temperature between 37° and 100° C at which N alkali begins to degrade DNA extensively to acid-soluble products. Sugiyama, Shinke and Ishida[45] incubated specimens of DNA in N potassium hydroxide for 5 hours at 50° C and observed that about 5 per cent. was then acid-soluble; a similar amount was released by incubation at 37° C for 15 hours. This suggests that degradation in N alkali is accelerated at some temperature above 50° C.

The stability of DNA to incubation in N alkali at 37° C has occasionally been challenged. The available evidence shows that DNA undergoes some depolymerisation above pH 11[115,116,117] to yield non-dialysable products of polynucleotide dimensions.[118] However, such depolymerisation does not necessarily result in less facile precipitation with acid; indeed, some reduction in molecular size has been claimed to make DNA more susceptible to acid precipitation.[119] Investigators who have looked for acid-soluble products after incubation of DNA in N alkali at 37° C for 15 to 18 hours have usually found no significant evidence of break-down. McCarter and Steljes,[21] Sugiyama, Shinke and Ishida[45] and Dutta[120] submitted samples of DNA to 15 to 17 hours' incubation in N alkali at 35° to 37° C and recovered some 95 to 98 per cent. by acid precipitation, and Wyatt[121] found no change in base ratios of two samples of DNA subjected to N sodium hydroxide for 18 hours at 37° C. In their original

publication, Schmidt and Thannhauser[9] described an experiment in which purified RNA and DNA were added to minced rat liver and were recovered quantitatively as additional phosphorus in the appropriate fractions after acidification of the alkaline digest. In a similar experiment involving addition of RNA and DNA to allantoic membranes, Graham[22] recovered 93 per cent. of the added phosphorus in the RNA fraction of the digest and 94 per cent. in the DNA fraction. Other authors have examined the acid-soluble portion of the alkaline digests of tissues for the presence of thymine derived from DNA and have recorded its absence after 18 hours of digestion in N potassium hydroxide at 30° C[71,122] or 37° C[123] or in 0·3 N potassium hydroxide at 30° C,[80] although Paigen and Kaufmann[124] observed appreciable amounts after 18 hours of digestion in N potassium hydroxide at 36° C. It may be observed that the last-named authors used heat to extract with lipid solvents after acid extraction, and this may well have degraded the DNA to apurinic acid. Tests for deoxyribose in the acid-soluble fraction of digests of liver have either been negative[30,35,125] or have yielded only traces.[23,32,126] Finally, Elson, Gustafson and Chargaff[95] used chromatographic procedures to separate the ribonucleotides in the acid-soluble fraction obtained after alkaline digestion of tissues and concluded that contamination of the ribonucleotides by break-down products from DNA could be excluded.

A few authors have claimed that DNA may not always be completely precipitated by acid after digestion in alkali under the conditions of the Schmidt - Thannhauser procedure. Most of these claims are debatable. In a widely quoted paper, Drasher[69] has castigated the Schmidt - Thannhauser method on the grounds that in some tissues the DNA may be degraded by alkaline digestion to a stage at which part is no longer acid-precipitable and so escapes estimation. This opinion is based on her measurements of DNA in mouse mammary tumour tissue, which gave considerably higher readings for deoxypentose when the tissue was extracted directly with hot TCA (Schneider[10] procedure) than on hot TCA extracts of the DNA fraction obtained by the Schmidt - Thannhauser procedure. It was therefore concluded that the Schmidt - Thannhauser value must be in error owing to loss of DNA into the acid-soluble RNA fraction, although no determinations of deoxypentose were made on this latter fraction. Drasher's direct measurement of the total phosphorus content of the Schneider extract falls considerably short of the amount computed by her to be present on the basis of the pentose and deoxypentose found in this extract. It thus seems a little ingenuous to claim that the deoxypentose values obtained on the Schneider extract are necessarily the correct ones merely on the grounds that they exceed the values obtained by the Schmidt - Thannhauser method. Nevertheless, the divergences reported by this author are of such a magnitude that anyone proposing to investigate the nucleic acid content of mammary tumours would be well advised to carry out careful checks on the adequacy of alkaline digestion for separating DNA from RNA.

Other groups of workers have reported the release of DNA break-down products into the Schmidt - Thannhauser RNA fraction obtained from cell nuclei. Thus Mauritzen, Roy and Stedman,[127] using isolated thymus nuclei, observed a positive Feulgen reaction and some thymine in the acid-soluble portion of the alkaline digest. However, their isolated nuclei were subjected to drying at room temperature for 2 days before being placed, without further chemical treatment, into N potassium hydroxide. It seems likely that break-down of DNA may have occurred enzymically during the drying period, with consequent appearance of the break-down products in the acid-soluble portion of the digest. More substantial evidence that calf thymus DNA may sometimes be degraded during alkaline digestion is provided by McIndoe and Davidson,[128] who subjected the Schmidt - Thannhauser RNA fraction obtained from thymus nuclei to ionophoretic separation and observed an ultra-violet absorbing area containing deoxyribose and of considerable magnitude. Since purified calf thymus DNA is stable to N alkali at 35° C for at least 17 hours,[21] it is possible that the observed break-down of DNA in the thymus nuclei could have been due to enzymic action during the period between removal of the thymus at the abattoir and chemical treatment of the specimen. It is significant that DNA break-down was much less evident in studies made in the same laboratory on rabbit thymus,[129] when the tissue would have been processed immediately after removal.

Two remaining claims to have observed degradation products of DNA in the RNA fraction after acidification of the Schmidt - Thannhauser alkaline digest can be regarded as insufficiently substantiated. In an analysis of the nucleic acid content of influenza virus, Graham[130] observed in addition to RNA a small amount of deoxypentose-reacting material,

most of it present in the RNA fraction after alkaline digestion; more extensive tests[46] have failed to confirm the presence of significant amounts of DNA in this virus. Finally, Tsanev and Markov[47] have suggested in a recent paper that a part of the DNA phosphorus can be split off during alkaline digestion and appear in the RNA fraction, even though deoxypentose and the bases remain in the fraction precipitated by acid. This conclusion is based on the lack of concordance of the authors' values for ultra-violet absorption, diphenylamine reaction and phosphorus content of the DNA fraction of the digest; as discussed later, this may have been due to inaccuracies in some of the methods used by them.

The question of DNA lability to alkali must be viewed in relation to the conditions causing formation of apurinic acid from DNA. When samples of DNA are incubated at 37° C in solutions at pH 1·6, the purine bases become completely released within 24 hours,[131] giving rise to apurinic acid. When this apurinic acid is subsequently treated with alkali, the hydroxyl groups exposed as the result of the loss of purine bases permit the formation of cyclic triesters under alkaline conditions, just as in RNA, and this is followed by break-down of tracts of the DNA chain to yield acid-soluble products. For example, exposure of apurinic acid to alkaline solutions as weak as pH 12·8 (0·06 N) for 24 hours at 37° C results in the release of 30 per cent. of its phosphorus in dialysable form.[132] Formation of apurinic acid can occur during the initial washing with cold acid to remove small molecules or during extraction with lipid solvents still contaminated with acid, if the temperature rises during these procedures. Bonar and Duggan[33] have studied the effect of raising the temperature, during the initial washing with acid to remove small molecules, on subsequent resistance of DNA to digestion by alkali. They exposed samples of DNA to 5 per cent. TCA, 0·2 N PCA or 0·2 N hydrochloric acid for 40 minutes, at room temperature, and subsequently treated the samples with N alkali for 1 hour at room temperature. Some 30 to 40 per cent. of the DNA became acid-soluble after alkaline digestion; when the preliminary treatment with 0·2 N PCA was carried out at 2° to 4° C, only 2 per cent. of the DNA was found in the acid-soluble fraction of the alkaline digest. Dutta[120] has also noted the influence of the initial treatment of a specimen of DNA on its resistance to subsequent hydrolysis by N alkali at 37° C over a period of 16 hours. Without any previous treatment, his specimens of purified liver DNA released about 5 per cent. of their phosphorus in acid-soluble form after treatment with alkali. Preliminary brief treatment of the DNA with 5 per cent. PCA in the cold resulted in 9 per cent. being solubilised during alkaline digestion. On the other hand, preliminary heating of the DNA to 100° C for 5 minutes made 25 per cent. of the DNA phosphorus susceptible to alkaline degradation. Evidence that apurinic acid can arise from DNA by heating specimens in lipid solvents still containing acid carried over from the initial acid washings has already been considered above, in the section on lipid extraction of tissues.

From the assembled data on DNA stability to alkali, it seems reasonable to conclude (*a*) that pure DNA is stable to N alkali at 37° C, and probably at temperatures considerably higher, although not as high as 100° C, (*b*) that many authors have successfully used the Schmidt - Thannhauser procedure on tissue samples without encountering evidence of DNA break-down and (*c*) that conditions introduced accidentally may lead to formation of apurinic acid and consequent susceptibility to alkaline degradation. Anyone setting out to use the Schmidt - Thannhauser procedure for the first time, or to use it on a new tissue, would therefore be well advised to test the RNA fraction for the presence of DNA (*e.g.*, by Ceriotti's[133] procedure for deoxypentose) and, if the test is positive, to explore the earlier stages of the determination for possible degradative effects on DNA.

Occurrence of insoluble residues at the end of alkaline digestion—Schmidt and Thannhauser[9] found that all tissues examined by them, with the exception of bone, were completely dissolved on exposure to N alkali at 37° C for 15 hours. However, some other authors,[19,24,134,135] using the same conditions of alkaline digestion, have observed a small residue of insoluble material. For animal tissues this seems not to be of importance. Davidson, Frazer and Hutchison[24] showed that the insoluble residue from alkaline digests of rabbit liver contained little phosphorus, and May and Grenell[19] found that the nucleic acid content of the insoluble residue from digests of brain corresponded to only 0·5 per cent. of the tissue DNA. On the other hand, the Schmidt - Thannhauser procedure can seriously underestimate the DNA content of some micro-organisms because of losses of DNA in alkali-insoluble residues. Part of the DNA of *Streptococcus faecalis* has been reported[123] to be insoluble in alkali through

being firmly bound to cell polysaccharides, and much of the DNA of Mycobacteria remains insoluble at the end of alkaline digestion in a residue probably lipoidal in nature.[136] Downing and Schweigert[137] report that two-thirds of the DNA of *Euglena gracilis* and of *Lactobacillus leichmanii* remains undissolved after treatment of the lipid-free material with N potassium hydroxide at room temperature for 20 hours, but Smillie and Krotkov[32] had no difficulty in dissolving all the nucleic acid of Euglena in N potassium hydroxide at 37° C. In Chlorella they observed an insoluble residue with no significant phosphorus content. In yeast cells submitted to the Schmidt - Thannhauser procedure, it has been reported[138] that there is an alkali-resistant polysaccharide residue containing a considerable amount of phosphorus, mostly in the form of metaphosphate.

It can be concluded that residues observed at the end of digestion of mammalian tissue preparations are not likely to constitute a serious hazard to analysis, but that, in micro-organisms, where the DNA is not confined within a nuclear structure, alkali-insoluble material is liable to retain significant amounts of DNA. In order to evaluate the significance of residues observed at the end of alkaline digestion, the total phosphorus content should be determined. Further, the residue can be extracted with hot TCA[136] or with hot PCA[19] and the extract examined for pentose and deoxypentose, by appropriate sugar reactions, and for ultra-violet absorption at several wavelengths.

METHODS USED FOR SEPARATING RNA AND DNA AFTER ALKALINE DIGESTION—

In the original Schmidt - Thannhauser[9] procedure, DNA and protein are precipitated at the end of alkaline digestion by adding hydrochloric acid and TCA. This particular mixture seems to possess no special virtues; indeed, when ultra-violet measurements have subsequently to be made, TCA should be avoided because of its high absorption. Other methods of acidification can be used, such as addition of hydrochloric acid,[139] acetic acid[109] or a mixture of acetic acid and ethanol,[140,141] the order of addition being important,[35] or TCA and ethanol.[142] When the production of an ion-free solution is an advantage, as when the ribonucleotides are subsequently separated by chromatography or ionophoresis, the specimen can be incubated with potassium hydroxide and the digest then acidified with PCA to remove the K^+ ions as the sparingly soluble potassium perchlorate (*e.g.*, Davidson and Smellie[107]; Scarano and Kalckar[143]; Schmidt[14]). An ion-free solution can also be obtained by acidifying the digest with a cation-exchange resin in the hydrogen form.[96,97] When the amount of DNA to be recovered is small, Schmidt, Hecht and Thannhauser[144] recommend addition of egg albumin to the alkaline digest in order to provide a bulky precipitate on acidification. On one occasion, when the DNA may have been partially degraded in the earlier stages of the determination, Smillie and Krotkov[32] found that addition of magnesium ions after acidification of the digest facilitated precipitation of the DNA, as suggested by Markham.[12]

The usual procedure is to acidify the digest so as to bring the pH down to 1 and then to remove by centrifugation the precipitated protein and DNA, finally washing the precipitate once or twice with dilute acid. The supernatant fluid and washings are combined and analysed for ribonucleotides, the precipitate being retained for measurement of DNA. The procedure of washing once or twice with acid appears to provide virtually complete separation of RNA from DNA, as judged by the failure of Daoust and Hooper[26] to extract more phosphorus from the DNA precipitate by further washing. Further, several investigators have found less than 1 to 2 per cent. of the total RNA of the tissue in the DNA fraction, as indicated by pentose assays[32,139] or by examination for uracil.[122] When ^{15}N-labelled yeast RNA was added to samples of rat liver, Furst, Roll and Brown[145] recovered 4 per cent. of the labelled RNA in the DNA fraction precipitated after alkaline digestion, but they have provided no details of their procedure for acid precipitation and washing of the precipitate. More serious contamination of the protein precipitate with RNA is claimed by Cooper and Loring,[146] who examined the acid-precipitable phosphorus after digesting a purified virus containing RNA; some 12 per cent. of the phosphorus of the digest could not be washed out of the precipitate; however, on re-dissolving the precipitate in alkali and precipitating again, this was reduced to 1 or 2 per cent.

Finally, procedures other than acidification can be used to separate the ribonucleotides from the DNA of the alkaline digest. Nygaard and Rusch[147] added 2 volumes of ethanol to the digest, and McIntire and Sproull[79] used protamine to precipitate a few μg of DNA from alkaline digests of tissue culture cells. Passage of the alkaline digest through ion-exchange columns also permits the ribonucleotides to be separated from the DNA.[148] The use of

paper electrophoresis to separate RNA from DNA has been explored,[149] but has not been placed on a quantitative basis.

THE RNA FRACTION OF THE ALKALINE DIGEST—

Determination of the phosphorus content of the RNA fraction—Schmidt and Thannhauser[9] considered that the RNA fraction obtained on acidifying the alkaline digest consisted of ribonucleotides contaminated by a "negligible" amount of inorganic phosphate, which, in view of the findings of Plimmer and Bayliss,[150] was considered to be derived from phosphoprotein. They therefore assumed that the phosphorus content of the fraction could be used as a measure of the amount of RNA in the tissue. Although such an assumption has serious limitations (see below), many investigators have so employed phosphorus determination, and it is therefore appropriate to consider briefly here the methods available.

Procedures for phosphorus analysis have been excellently reviewed by Lindberg and Ernster[151] and are partly covered by LeLoir and Cardini.[152] Because of the small amount of phosphorus present in the RNA fraction, classical gravimetric procedures are generally not applicable, and the first step commonly involves conversion of organic phosphorus to inorganic orthophosphate by wet ashing of the nucleic acid fraction with sulphuric acid,[153] PCA[154,155] or a mixture of sulphuric acid, copper sulphate, potassium hydrogen sulphate and selenium dioxide,[156] first used by Ma and Zuazaga.[157] Digestion with PCA is occasionally explosive and should be carried out with caution. It is worth noting that Jones, Lee and Peacocke[156] observed that, if the digestion of DNA were carried out with PCA alone, a period of up to 7 hours was necessary for the quantitative conversion of the DNAP to orthophosphate, whereas with the Ma and Zuazaga[157] mixture digestion was complete in 45 minutes. The orthophosphate produced by the ashing process is allowed to react with molybdic acid to produce a complex, which can be reduced to a blue compound. Although in the original method of Briggs[158] the use of molybdic acid was recommended, most subsequent variants involve use of ammonium molybdate solutions; similarly, the use of hydroquinone by Briggs[158] for the final reduction has been supplanted by a variety of compounds such as 1-amino-2-naphthol-4-sulphonic acid,[153,159,160] amidol (2,4-diaminophenol hydrochloride)[155,161] and stannous chloride.[162,163]

The main problem confronting the worker in the nucleic acid field is that of finding a method adequately sensitive at the low concentrations of phosphorus encountered. For many purposes, the Allen[155] procedure is adequate, especially if the volumes used are scaled down to give a final volume of 5 ml, so that the range of the method is between 4 and 40 μg of phosphorus. When this is inadequate, recourse may be had to methods involving extraction of the blue complex into an organic solvent[159,163] or heating to increase the colour intensity.[108,160] An ultra-micro method, based on the colour change of quinaldine red, has also been described[164] and has been claimed to have a range of 0·02 to 0·2 μg of phosphorus per ml, 15 times as sensitive as the Fiske - Subbarow procedure.

Several authors have explored the problem of whether or not the amount of phosphorus present in the RNA fraction of the alkaline digest adequately represents the true RNA content of the tissue. Schmidt and Thannhauser[9] considered that the only non-RNA phosphorus present was a "negligible" quantity of inorganic phosphate arising from the break-down of phosphoprotein in the alkali.[150] Davidson, Frazer and Hutchison,[24] Moulé[23] and Annau[77] confirmed that contamination of this type was small. However, accumulated evidence points to the presence in the RNA fraction of considerable amounts of organic phosphorus compounds other than ribonucleotides. For nervous tissue Logan, Mannell and Rossiter[17] showed that 50 to 65 per cent. of the phosphorus present in the RNA fraction of the alkaline digest was not RNA phosphorus. In the same year, Davidson and Smellie[165] demonstrated that the RNA fraction from rat liver contained at least six phosphorus compounds (including inorganic phosphate) other than ribonucleotides. This material amounted to some 20 to 25 per cent. of the phosphorus in the fraction. Subsequent investigation[166] showed that several of these contaminants are peptide in nature and that phosphoserine is present as well. The RNA fraction was found to contain also free inositol, inositol monophosphate and, from brain, inositol diphosphate. In the RNA fractions obtained by alkaline digestion of micro-organisms, other phosphorus-containing contaminants have been observed. Working with yeast Juni, Kamen, Reiner and Speigelman[138] observed that 67 per cent. of the combined phosphorus of the RNA fraction was hydrolysable in 7 minutes by N acid at 100° C and was identified as metaphosphate. Mitchell and Moyle[167,168,169] and Jones, Rizvi and Stacey[170]

have reported the occurrence of glycerophosphate as a contaminant in the RNA fraction obtained from bacteria. In animal tissues, there appears to be neither metaphosphate[23,24] nor glycerophosphate.[166]

The presence of appreciable amounts of non-RNA phosphorus in the RNA fraction of the alkaline digest also emerges when the phosphorus content of this fraction is compared with the results of other procedures for determining its RNA content. In Table II, results

TABLE II

SCHMIDT - THANNHAUSER RNA FRACTION ANALYSES CALCULATED ON THE BASIS OF THE PHOSPHORUS CONTENT AS 100 per cent.

Species	Tissue	Orcinol	Ultra-violet absorption	Author and reference
Rat	Liver	85	—	Schneider[171]
		85*	—	Campbell and Kosterlitz[172]
		75	—	Moulé[173]
		124	68†	Tsanev and Markov[47]
Rat (pregnant) ..	Liver	86*	—	Campbell and Kosterlitz[172]
Rat	Liver nuclei	80	84†	McIndoe and Davidson[128]
Rat (fasted) ..	Liver nuclei	84	87†	McIndoe and Davidson[128]
Rat	Kidney	79	—	Schneider[171]
		79	—	Moulé[173]
	Pancreas	92	—	Schneider[171]
		95	100	Mandel, Jacob and Mandel[174]
		71	—	Moulé[173]
	Spleen	87	—	Schneider[171]
		75	—	Mandel, Jacob and Mandel[174]
	Thymus	90	—	Schneider[171]
	Adrenal	64	73	Mandel, Jacob and Mandel[174]
	Brain	59	—	Schneider[171]
Mouse	Liver nuclei	84	—	Nash[88]
	Mammary tumour	60	—	Drasher[69]
	Skin	155	78†	Tsanev and Markov[47]
	Guérin carcinoma	143	82†	Tsanev and Markov[47]
Guinea pig ..	Seminal vesicle	95	100†	Humphrey and Kellerman[175]
Rabbit	Liver	86	—	Davidson, Frazer and Hutchison[24]
	Liver nuclei	72	—	McIndoe and Davidson[126]
Dog	White matter	25	—	Moulé[173]
	Grey matter	57	—	Moulé[173]
Calf	Thymus nuclei	67	91†	McIndoe and Davidson[126]
Fowl	Liver	82	98	Moulé[23]
		79	—	Moulé[173]
	Spleen	60	92	Moulé[23]
		64	—	Moulé[173]
	Erythrocyte nuclei	43	—	McIndoe and Davidson[128]
Sea urchin ..	Embryo	84	91†	Elson, Gustafson and Chargaff[95]
Chlorella		71	46	Smillie and Krotkov[32]
M. pyogenes		—	56	Mitchell and Moyle[168]
M. pyogenes var. *aureus*		—	70	Mitchell and Moyle[167]

* Phloroglucinol method used.
† Based on ultra-violet absorption at two wavelengths.

obtained on a variety of biological materials by the orcinol or phloroglucinol procedures and by ultra-violet absorption are expressed as percentages of the total phosphorus content of the RNA fraction. As discussed later, both pentose and ultra-violet determinations of RNA in this fraction can involve errors that usually cause over-estimation of the RNA present. Nevertheless, it is a striking feature of the results recorded in Table II that, except for the high orcinol values observed by Tsanev and Markov,[47] all the pentose and ultra-violet results show a considerably lower value than the phosphorus content of the RNA fraction would appear to demand. The discrepancy is particularly large for fowl erythrocyte nuclei and nervous tissue. The RNA contents of the alkaline digest assessed by other methods are consistent with the picture provided by Table II. With *Streptococcus faecalis*, Sherratt and Thomas[123] showed that the sum of purine and pyrimidine bases accounted for 85 per cent. of the total phosphorus present in the RNA fraction of the alkaline digest; for tobacco-leaf chloroplasts Cooper and Loring[55] observed that the purine and pyrimidine contents of the RNA fraction were sufficient to account for only 91 per cent. of the total phosphorus in this

fraction. As discussed earlier, the ionophoretic studies of Davidson and Smellie[165] on the RNA fraction of rat liver led to the conclusion that only 75 to 80 per cent. of the phosphorus in this fraction could be accounted for as ribonucleotides.

Since phosphorus determinations made on the RNA fraction of the alkaline digest overestimate the true RNA content for most tissues by some 20 per cent., and occasionally by much larger amounts, alternative methods of measuring RNA contained in this fraction must be considered. They include determination of pentose, determination of ultra-violet absorption and isolation of nucleotides or bases from the RNA fraction.

Determination of the pentose content of the RNA fraction—A number of colorimetric procedures for pentose have been applied to the determination of RNA, namely, reaction with orcinol, phloroglucinol, aniline, carbazole, cysteine or anthrone. Of these, the commonest is the orcinol reaction of Bial.[176] This was first applied to analysis of nucleotides by Dische and Schwarz[177] and has since undergone numerous modifications, the most widely quoted being that of Mejbaum.[178] The applications of the orcinol procedure have been summarised in a number of review articles.[11,12,179,180,181,182,183] The orcinol determination of ribose will here be considered in relation to (*a*) the optimum concentration of reactants and the time of heating, (*b*) the extent of reaction of ribose in RNA with orcinol and (*c*) the occurrence in biological materials of substances other than ribose that can react with orcinol.

The orcinol reaction depends on the hydrolysis of the nucleic acid by acid at 100° C to yield furfural from the ribose. This then reacts with orcinol to yield a green pigment. In the original procedure, ferric chloride was used as the catalyst, and most workers have continued to employ this, but some have used ammonium ferric sulphate,[184,185] and a few, such as Ceriotti,[39] have followed the practice of Barrenscheen and Peham[186] in using cupric chloride. Finally, some have extracted the green solution with butanol[187] or isopentyl alcohol,[39,188] with consequent increase in the sensitivity of the method through concentration of the pigment. In the original procedure of Dische and Schwarz,[177] the reagents were added to give a final concentration of 7·7 N hydrochloric acid, 0·06 per cent. of ferric chloride ($FeCl_3.6H_2O$) and 0·14 per cent. of orcinol, and these were heated with the specimen for 3 minutes at 100° C. Table III shows that the concentrations of all three reagents and the

TABLE III

SOME VARIATIONS IN THE CONDITIONS OF THE ORCINOL REACTION FOR PENTOSE (IN ORDER OF PUBLICATION)

Final normality of hydrochloric acid*	Final concentration of $FeCl_3.6H_2O$, %	Final concentration of orcinol, %	Time of heating, minutes	Author and reference
7·7	0·06	0·14	3	Dische and Schwarz[177]
6·0	0·05	0·50	20	Mejbaum[178]
6·0	0·05	0·50	30	Schlenk[189]
5·8	0·01	0·29	20	Kerr and Seraidarian[190]
7·1	0·06	0·15	20	McRary and Slattery[191]; Brown[192]
7·1	0·15	0·15	3	Militzer[187]
5·7	0·05	0·48	45	Albaum and Umbreit[193]
6·0	0·50	0·50	40	Schlenk and Waldvogel[194]
5·7	0·03†	0·48	45	Drury[184]
5·7	0·48	0·48	45	Beljanski and Macheboeuf[195]
8·2	0·01	0·41	20	Morse and Carter[196]
4·0	0·04	6·00	30	Caldwell and Hinshelwood[197]
5·7	0·02	0·48	11	Ogur and Rosen[16]
6·0	0·05	0·50	60	Lusena[198]
8·0	0·03	0·27	15	Hurlbert, Schmitz, Brumm and Potter[199]
6·0	0·01	0·48	45	Rege and Sreenivasan[200]
8·6	0·18	0·18	8	Markham[13]
5·7	0·05	0·48	40	Ashwell[182]
8·2	0·02	0·41	20	Iverson and Giese[24]
6·0	0·25	0·50	20	Schneider[181]
9·0	0·015	0·015	15	Stuy[201]
6·0	0·01	0·30	30	Ramaiah, Antoni and Hutchison[202]

* Based on the assumption that when the author states that concentrated hydrochloric acid was used this means 12 N hydrochloric acid.

† $Fe(NH_4)(SO_4)_2.12H_2O$ calculated as $FeCl_3.6H_2O$.

time of heating have been extensively varied by different users of this reaction. Miller, Golder and Miller[203] made a systematic study of the effect of different conditions of reaction on the final colour developed with RNA. This was increased by raising the concentrations of acid, iron or orcinol up to a certain maximum level, after which further increments in reagent concentration resulted in less colour being formed. The rate at which the colour developed could be accelerated by increasing the concentration of orcinol or of acid, but was relatively uninfluenced by the concentration of iron. Under optimal conditions, the reaction with RNA was essentially complete after 30 minutes' heating at 100° C. Miller, Golder and Miller[203] point out that the choice of suitable conditions for carrying out the reaction is dictated by two opposing considerations: with increasing amounts of reagents, colour development rises to a maximal value, but at the same time the reading of the blank is also augmented; they concluded that the conditions used by Drury[184] (see Table III) provide the best compromise. We have found that the somewhat lower concentrations of orcinol and of iron used by Kerr and Seraidarian[190] are satisfactory if the heating time is prolonged to 30 minutes.[202] In agreement with Kerr and Seraidarian,[190] we found the colour formed under their conditions not to fade appreciably for several hours; some authors working under different conditions[192,199] observed definite fading over short periods of standing after completion of the reaction.

The reaction of orcinol with the ribose of RNA is complicated by the fact that the purine-bound ribose of RNA reacts much more readily than pyrimidine-bound ribose. The latter is not completely unreactive, however, and has been observed to give a colour representing from 3 to 10 per cent. of the ribose attached to the pyrimidine.[195,204,205] As pointed out by Webb and Levy,[15] large differences in purine - pyrimidine ratio can sometimes exist between different species of RNA, extremes of 2·13 for *Mycobacterium phlei*[206] and 0·66 for turnip yellow mosaic virus[207] being recorded. Consequently, the value to be assigned to the result obtained from the orcinol reaction and other carbohydrate reactions for RNA will depend on the proportion of purine and pyrimidine nucleotides in the molecule; the standard used should ideally consist of pure RNA isolated from the tissue on which the determinations are being carried out. In this connection, Massart and Hoste[208] offer a method of obtaining full reaction from the pyrimidine-bound pentose by first brominating the pyrimidine ring before submitting these nucleotides to reaction with orcinol; however, Kerr, Seraidarian and Wargon[209] were unable to apply this procedure successfully to RNA.

Like other sugar reactions, the orcinol reaction suffers from lack of complete specificity. Interference has been noted from the presence of hexoses,[192,210,211,212] sucrose,[213] pentosans[16,214,215] polyuronides,[16,210] heptoses and their phosphates,[183,212] polysaccharides,[193] glycogen,[210,216] large amounts of protein,[179,194,210] phosphates,[169] TCA[39] and DNA.[181] In some instances methods have been devised for dealing with the interference; for hexoses, these include extraction of the interfering substance,[192] taking readings at two wavelengths[192] or destruction of the hexose by heating in alkali.[211] Interference by protein can be eliminated by extracting the coloured product with pentyl alcohol.[194] Many of the potential causes of error in the orcinol determination of ribose have not been examined in the context of nucleic acid analysis.

With the Schmidt - Thannhauser procedure, only a few authors have reported difficulties in applying the orcinol reaction to the RNA fraction. Juni, Kamen, Reiner and Spiegelman[138] observed a brown colour when the orcinol reaction was applied to the RNA fraction of yeast, presumably due to the presence of polysaccharide; the amount of this contaminant was sufficient to render the orcinol reaction valueless as a means of determining RNA. Smillie and Krotkov[32] observed the occurrence of orcinol-reacting polysaccharide in the RNA fraction obtained from plant material; acceptable orcinol values could be achieved by first passing the RNA fraction through an anion-exchange column to remove the contaminants. Sato[142] used 4 volumes of ethanol to precipitate polysaccharide material from the RNA fraction of bacteria. In contrast to these experiences with plant materials, Table II shows that direct application of the orcinol reaction to the RNA fraction obtained from animal tissues has generally provided slightly lower estimates of RNA content than those obtained by ultra-violet measurements on the same fraction, thus suggesting the absence of gross contamination by orcinol-reacting substances other than RNA. An exception to this are the high orcinol values noted by Tsanev and Markov[47] in the RNA fraction of animal tissues, including liver, which do not accord with the experience of others quoted in Table II. The conclusion that large amounts of orcinol-reacting impurities are confined to digests of

yeast and plant materials receives some support from the studies of Deken-Grenson and Deken.[217] They used Dowex-2 resin to remove orcinol-reacting impurities from the RNA fraction of various tissues submitted to alkaline digestion, and it is apparent from their results that large changes in orcinol value after purification occurred with plant material and yeast specimens, whereas the values obtained for RNA fractions derived from bacteria and from liver were only slightly reduced (5 to 10 per cent.) by treatment with the resin. Fleck and Munro[38] have made a more detailed study of the orcinol reaction applied to the RNA fraction of liver after various conditions of alkaline digestion. When the specimens were digested for 1 hour at 37° C in N or 0·3 N alkali, all the RNA of the tissue became acid-soluble and the orcinol reaction provided a reasonable measure of the amount of RNA present. After 24 hours' digestion in alkali, the orcinol value had risen by about 8 per cent., and this coincided with the release of protein into the acid-soluble fraction of the digest. It thus appears that prolonged digestion in alkali causes release of some orcinol-reacting impurities and thus increases the error of determining RNA.

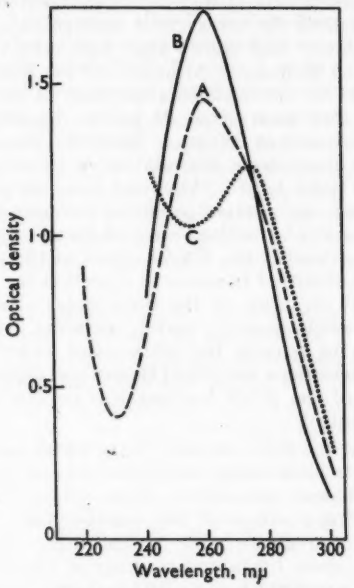

Fig. 3. Ultra-violet absorption spectra in 0·1 N PCA of : curve A, pure rat liver RNA after hydrolysis in 0·3 N potassium hydroxide for 18 hours at 37° C; curve B, commercial yeast RNA after hydrolysis under similar conditions; curve C, the polypeptide material appearing in the Schmidt - Thannhauser RNA fraction. The spectra of all three have been adjusted by calculation to give the same optical density at 275 mμ, which is the maximum absorption of the polypeptide. (Reproduced from Fleck and Munro[38])

Alternative reactions to the orcinol procedure have been used for determining ribose, but none has been so extensively explored. The reaction with phloroglucinol,[210,212,218] although more specific than that with orcinol, requires about five times the amount of ribose. The furfural formed by heating RNA with strong mineral acid can be trapped in xylene or by distillation and then made to react with aniline acetate,[127,142,219,220] *p*-bromoaniline[221] or *p*-bromophenylhydrazine.[222] These procedures are all more complicated to perform than

those of the orcinol method. The carbazole[223,224] and cysteine[225,226,227] reactions are more commonly used to assay DNA, but under suitable conditions can be applied to RNA; both reactions are subject to interference by other sugars. Finally, the anthrone reagent, originally developed for the determination of hexoses,[228] has been successfully adapted to determining pentoses,[229,230,231,232,233] but there appear to be no special advantages in its use.

Determination of the ultra-violet absorption of the RNA fraction—The intense and characteristic absorption of ultra-violet light by purine and pyrimidine bases in the region of 260 mμ provides a method of measuring the ribonucleotides in the acid-soluble fraction produced by the Schmidt - Thannhauser procedure, but the use of ultra-violet absorption is complicated by the fact that proteins and peptides also absorb ultra-violet light at similar wavelengths (Fig. 3). Several authors[38,47,127,166,234,170,217] have shown the Schmidt - Thannhauser RNA fraction to contain protein-degradation products that absorb ultra-violet light. Consequently determination of the RNA content of this fraction by ultra-violet absorption at 260 mμ will give erroneously high readings. The magnitude of the error can be considerable under the usual conditions of digestion continued for 15 hours or more. Scott, Fraccastoro and Taft[73] measured the ultra-violet absorption of the RNA fraction obtained from specimens of liver, kidney and spleen that had been digested in N alkali at room temperature for periods up to 24 hours. Although all the RNA became acid-soluble during the first hour of incubation, the ultra-violet absorption of the RNA fraction continued to rise; inspection of their graphs shows that 24 hours' digestion contributed an additional 15 to 25 per cent. to the absorption at 260 mμ. Fleck and Munro[38] confirmed that continued alkaline digestion results in progressive degradation of tissue protein, yielding acid-soluble products that absorb ultra-violet light. When rat-liver samples were digested for 1 hour in 0·3 N alkali at 37° C, these degradation products accounted for about 1 per cent. of the ultra-violet absorption of the RNA fraction; after 24 hours' digestion in N alkali ultra-violet absorption at 260 mμ over-estimated the RNA content of the fraction by about 35 per cent. Jones, Rizvi and Stacey[170] submitted bacteria to digestion for 15 hours in N alkali at 37° C and observed that about 30 per cent. of the ultra-violet absorption of the RNA fraction was due to non-nucleotide contaminants, mainly peptides. Deken-Grensen and Deken[217] used an ion-exchange resin to remove the ultra-violet absorbing contaminants from the RNA fraction prepared by digesting a variety of tissues and organisms in N alkali for 20 hours; the ultra-violet absorption of the RNA fraction was on occasion reduced by as much as 70 per cent. after this treatment.

These findings show that the RNA content of the tissue can be seriously over-estimated by measuring at 260 mμ the ultra-violet absorption of the acid-soluble fraction obtained during the Schmidt - Thannhauser procedure. Some authors have attempted to correct for the extra absorption by taking readings at two wavelengths. The spectra of RNA and of the contaminating peptide material differ (Fig. 3), and it is consequently possible to compute the amount of RNA in a mixture by taking readings at the maximum absorption of RNA (260 mμ) and at one other wavelength (*e.g.*, 280 mμ) and applying an equation based on the ultra-violet absorption of RNA and of the contaminant material at each of these wavelengths.[38] This principle was first applied by Warburg and Christian[235] to measure the amount of RNA in mixtures of yeast RNA and enolase, under conditions in which the spectra of both the RNA and the enzyme protein had been adequately established. A special instance of this principle occurs when it is possible to choose two wavelengths at which the peptide contaminant absorbs the same amount of ultra-violet light; for example, Fig. 3 shows the spectrum of non-dialysable peptide material prepared from the acid-soluble fraction of liver samples digested in N alkali, and it is apparent that this material absorbs the same amount of ultra-violet light at 284 mμ as at 260 mμ. Consequently, if the amount of ultra-violet light absorbed by the acid-soluble fraction of the liver digest is measured at these two wavelengths, any excess absorption at 260 mμ over the amount of light absorbed at 284 mμ can be ascribed to the presence of RNA. It is presumably for this reason that some authors measuring the ultra-violet absorption of the RNA fraction of the alkaline digest have preferred to use the difference between the absorptions at 260 and 280 mμ[175] or at 260 and 290 mμ,[80,95,128] though in none of these instances was it established that the choice of wavelengths completely eliminated absorption by peptides. A rigorous examination of the wavelengths required to provide these conditions has recently been made by Tsanev and Markov,[47] who concluded that the peptides contaminating the acid-soluble fractions from

a wide variety of tissues had essentially the same spectrum. In order to eliminate absorption by these contaminants, Tsanev and Markov digest the tissue in N alkali at 37° C for 18 hours and take readings of the absorption of the acid-soluble fraction at 260 and 286 mμ. However, Fleck and Munro[38] have pointed out that the procedure lacks precision, because (*a*) the method of obtaining the spectrum of acid-soluble peptide material is not above criticism, (*b*) the absorption of RNA at 286 mμ relative to its absorption at 260 mμ is not constant for different tissues, as Tsanev and Markov assume, but varies considerably as is shown for yeast RNA and liver RNA in Fig. 3 and (*c*) the RNA absorption at 286 mμ will depend on the degree of deamination of cytidylic acid in N alkali at 37° C, referred to earlier. Fleck and Munro[38] attempted to find conditions under which a reliable correction for protein-degradation products could be obtained, but were unable to do so. They concluded that the use of ultra-violet absorption to measure RNA by the Schmidt - Thannhauser procedure should be restricted to analyses involving short periods of digestion, since this results in least contamination of the RNA fraction by acid-soluble peptide material. Several authors[33,38,73,74] have demonstrated that a 1-hour period of digestion in 0·3 N or N alkali is sufficient to release all the RNA from a variety of tissues in an acid-soluble form. This would therefore seem a suitable period of digestion to choose. Ultra-violet absorption measurements made on RNA fractions prepared in this way will not show anomalous readings because of the hyperchromic effect produced by depolymerisation in alkali,[236,237,238] since 1 hour's digestion in 0·3 N alkali at 37° C is adequate to carry the hyperchromic effect to completion.[38]

Quantitative separation of bases and nucleotides in the RNA fraction—In theory, an unequivocal value for the amount of RNA in the acid-soluble portion of the alkaline digest can be obtained by quantitative isolation of the individual nucleotides or bases present in the RNA fraction. This may be attempted (*a*) by separating the ribonucleotides already liberated during alkaline digestion, (*b*) by degrading the ribonucleotides with concentrated PCA to yield free bases, which can then be separated, or (*c*) by using mild acid hydrolysis to provide a mixture of free purine bases and pyrimidine nucleotides, which are then separately assayed.

Ribonucleotides released by alkaline digestion have been separated quantitatively by paper-chromatographic procedures[95,110,239] and by ionophoresis.[107,165,240] The products of alkaline digestion have also been separated on ion-exchange columns,[241] and this procedure has been used to recover ribonucleotides quantitatively.[76,103,148] Finally, Montreuil and Boulanger[78] have used a technique involving partial separation of nucleotides on a De-Acidite column and paper chromatography. All such procedures involve inherent sources of error, for, as mentioned earlier, there is no guarantee of complete resolution of RNA to mononucleotides by the alkaline-digestion procedure of Schmidt and Thannhauser. For example, Finamore and Volkin[103] have recently recorded the presence of 10 and 20 per cent. of alkali-resistant oligonucleotides in two fractions of RNA extracted from amphibian eggs.

Conditions suitable for liberating free bases from RNA have been reviewed by Wyatt.[242] The most commonly used procedure is that of Marshak and Vogel,[243] in which the specimen of RNA is digested in 72 per cent. PCA at 100° C for 1 hour and the free bases are then separated by paper chromatography. Several authors[123,124,201] have applied this procedure to analysis of the RNA fraction from the Schmidt - Thannhauser method. However, it has been repeatedly noted[35,244] that the method of digestion with concentrated PCA does not liberate pyrimidine bases quantitatively from the corresponding nucleotides, especially in presence of protein.[245] Markham[12] recommends a 2-hour period of hydrolysis for complete liberation of bases. The chromatographic separation of the free bases from the charred material left after PCA digestion can also make this procedure difficult to control.

Loring[246] has reviewed the conditions under which RNA can be hydrolysed by acid to yield purine bases and pyrimidine nucleotides. When RNA is heated at 100° C in N hydrochloric acid for 1 hour, the purine bases are completely liberated as free adenine and guanine, whereas the pyrimidine bases remain in the form of nucleotides, except for about 5 per cent. degraded to nucleosides.[207] These conditions of mild acid hydrolysis have been applied to analysis of the RNA fraction obtained after alkaline digestion. Vendrely[134,247] precipitated the purine bases as their copper salts and determined the nitrogen. A more elegant procedure is to separate the digest into two fractions, one purine and the other pyrimidine; the amounts of adenine and guanine or cytosine and uracil are then determined by measuring the ultra-violet absorption of each fraction at two wavelengths. In order to obtain independent

purine and pyrimidine fractions, Kerr, Seraidarian and Wargon[209] precipitated the purine bases as their silver salts, with subsequent precipitation of the silver derivatives of the pyrimidine nucleotides. Loring and his colleagues[55,248] also precipitated the purine bases as silver salts, but degraded the pyrimidine nucleotides with phosphatase to nucleosides before measuring their ultra-violet absorption; they removed ultra-violet absorbing amino acids from the pyrimidine nucleoside fraction by passing it through Dowex-1 resin. Abrams[249,250] describes a modification in which labelled purines are added to the RNA fraction and the amount of adenine and guanine present in the silver precipitate is measured by isotope dilution. Finally, Kleinschmidt and Manthey[35] have devised a modification of the Schmidt - Thannhauser procedure in which, after hydrolysing the RNA fraction with acid, they fractionate the purine bases and pyrimidine nucleotides by two-dimensional paper chromatography; they do not mention the production of pyrimidine nucleosides during their assay procedure.

All of these procedures for obtaining quantitative recovery of bases or nucleotides are technically more complicated than the common procedures for measuring RNA, and most are unsuited to use for analysing a large number of samples. They may be kept in reserve for conditions in which other analytical procedures have not led to acceptable values for RNA. Even so, their limitations should be clearly recognised. This is brought out by a comparison of different methods of measuring the purine and pyrimidine components of RNA made by Crosbie, Smellie and Davidson.[244] They analysed a specimen of yeast RNA by chromatography of the bases after digestion in 72 per cent. PCA, by paper chromatography of purine bases and pyrimidine nucleotides after hydrolysis in N hydrochloric acid and by ionophoretic separation of nucleotides after alkaline hydrolysis. Recoveries corresponding to 86, 89 and 97 per cent., respectively, of the phosphorus content of the RNA specimen were obtained by the three procedures.

THE DNA FRACTION OF THE ALKALINE DIGEST—

The fraction of the alkaline digest precipitated on acidification contains the DNA of the tissue and most of its protein. In order to analyse the precipitate for DNA, it can be dissolved in alkali.[24] Alternatively, in Schneider's[171] modification of the Schmidt - Thannhauser procedure, the fraction is extracted with hot acid in order to obtain the DNA free from contamination with the protein, which would interfere with some colorimetric reactions for deoxypentose and with ultra-violet measurement of DNA. The extraction is usually done with 5 per cent. TCA or with 5 per cent. (0·8 N) PCA at 90° C for 15 minutes.[11] Davidson[129] recommends extraction with 3 per cent. (0·5 N) PCA at 80° C for 10 minutes. A number of authors have encountered difficulties with the Schneider modification. Using TCA, Drasher[69] found that shortening of the time of extraction led to reduced recovery of DNA, and Badolato and Calabrese[125] observed that TCA was much less effective than PCA in extracting DNA. Marshak and Marshak[122] record an 85 per cent. recovery of thymine extracted with hot TCA from the DNA fraction of echinoderm germ cells. On the other hand, the use of PCA at 90° C has been found to result in destruction of some deoxypentose, since higher values were obtained when the temperature of extraction was reduced to 70° C.[217] In using the extract for ultra-violet measurement of DNA, it has been found impossible to obtain conditions under which hot PCA will remove all the DNA from the precipitate without also extracting some of the protein and in consequence giving rise to erroneously high ultra-violet readings.[73,251] Because of these findings, it would seem preferable to dissolve the DNA fraction in alkali as a preliminary to analysis.

As with the RNA fraction, the amount of DNA present can be determined by measuring the phosphorus, from the sugar component (deoxypentose), by ultra-violet measurement or by isolation of the bases or nucleotides. Each of these will be considered separately.

Determination by phosphorus content—Schmidt and Thannhauser[9] originally measured the DNA by subtracting the phosphorus content of the RNA fraction from the total amount of phosphorus in the alkaline digest, but Schmidt, Hecht and Thannhauser[144] modified the procedure so that the phosphorus content of the DNA precipitate was measured directly. A number of authors have reported good agreement between the phosphorus method for DNA and the results of deoxypentose measurements or ultra-violet absorption made on the DNA fraction directly[5,30,128,175,252]; agreement between these three methods has also been reported on hot acid extracts of the DNA fraction.[17,23,171,253,254] Two authors,[47,125] however, obtained considerably greater values for deoxypentose or ultra-violet measurements than

by phosphorus determination, and Tsanev and Markov[47] have interpreted this as indicating partial lability of the DNA phosphorus during alkaline digestion; these findings and the conclusion drawn from them seem to be out of line with the satisfactory agreement found by the several other authors quoted above. Bourdet, Mandel and Guillemet[255] obtained for yeast lower values by diphenylamine than by phosphorus determination.

Determination by deoxypentose content—Several authors have reviewed in detail the available methods for deoxypentose.[15,180,181,182] Deoxypentose can be measured by reaction with diphenylamine, cysteine, indole, tryptophan, phloroglucinol, carbazole, *p*-nitrophenylhydrazine or anthrone. The reaction with Schiff's reagent, developed by Feulgen and Rossenbeck[256] for histochemical identification of DNA, is not adequately sensitive for determinations on tissue extracts.

The most frequently used method is the photo-electric adaptation[257] of the diphenylamine reaction of Dische,[223] which is suitable for the determination of 50 to 500 μg of DNA per sample or in one modification 25 to 250 μg of DNA[258] and 1 to 2 μg of DNA as a micro modification.[259] The sample is heated with diphenylamine in a mixture of glacial acetic acid and sulphuric acid for 10 minutes; Ishida[260] claims that longer periods of heating are necessary to produce a stable colour, but other authors have been satisfied with the colour stability after 10 minutes' heating.[182] Recently, Burton[261] has introduced a modified diphenylamine reagent containing acetaldehyde; the samples are heated at 30° C with this reagent for several hours. This procedure is about 3½-times as sensitive as the usual diphenylamine procedure, and Nigon and Daillie[262] describe a micro method suitable for samples containing 0·3 to 1·8 μg of DNA. The chemistry of the diphenylamine reaction has been studied by Stacey and his colleagues.[263,264,265,266] It appears that only the purine-bound deoxyribose contributes significantly to the colour produced when diphenylamine reacts with DNA. Brady and McEvoy-Bowe[267] propose a method involving preliminary bromination, which allows the pyrimidine-bound sugar to react as well.

Various substances are known to interfere with the diphenylamine reaction. In general, though most sugars react with diphenylamine, the colour produced differs from that with deoxyribose, and some of these spurious colour reactions can be eliminated by reading the absorbance of the final solution at two wavelengths.[180] These sugars are not likely to constitute a serious hazard in nucleic acid determinations under the usual conditions, although in one study of yeast by the Schmidt - Thannhauser procedure the DNA fraction gave an atypical green colour with the diphenylamine reagent,[138] presumably due to the presence of another carbohydrate in the DNA fraction. A more likely hazard in applying the diphenylamine reaction to the DNA fraction of the alkaline digest is the finding[218,268,269,270] that many proteins react with diphenylamine to give a colour. In studies on artificial DNA - protein mixtures, this source of error has been much reduced though not completely eliminated by extracting the DNA with hot TCA.[218,268,270,271] Since the DNA fraction from the Schmidt - Thannhauser procedure contains most of the protein of the tissue, the application of the diphenylamine reaction directly to it should lead to erroneously high readings. In fact, reasonable agreement with phosphorus values has been reported by all authors who have applied both determinations directly to DNA fractions prepared from animal tissues.[5,175,252] Further, Cohen[272] has reported identical colour intensities per unit of phosphorus when the diphenylamine reaction was applied to thymus nucleohistone and to purified DNA. Bergold and Pister[273] even dispute the finding of von Euler and Hahn[218] that addition of serum albumin to samples of DNA enhances the colour formed after reaction with diphenylamine. With his modified diphenylamine procedure, Burton[261] found that addition of a casein hydrolysate to DNA reduced the colour developed, in spite of the fact that casein hydrolysate gives a small amount of colour by itself. A claim that certain amino acids and purines can give spurious colours with diphenylamine[274] has not been confirmed.[269]

The reaction of deoxyribose with indole, first described by Dische,[275] has been modified by Ceriotti[39,133] to yield a method ordinarily suited to the measurement of as little as 5 μg of DNA and down to 0·2[276] or 0·1 μg[277] in micro modifications. The sample of DNA is made to react with indole in 3 N hydrochloric acid for 10 minutes at 100° C to give a yellow colour with a sharp absorption peak at 490 mμ. The reaction mixture is then extracted with chloroform, which leaves the yellow product from deoxyribose in the aqueous phase, but extracts completely the yellow or red pigments formed by other sugars and interfering substances that react in this test; only arabinose among the common sugars yields a colour

not fully extracted by the chloroform treatment, but this is unlikely to be present in animal tissue preparations being analysed for DNA. Keck[277] found that the arabinose error could be eliminated by using pentyl acetate in place of chloroform as the extractant. Bonting and Jones[276] suggest that the tedium of the chloroform extraction can be reduced by substituting one extraction with 10 volumes of chloroform in place of the usual three extractions with 1 volume. In addition, the elaborate purification of the chloroform, which was originally recommended by Ceriotti,[133] has been found by us to be unnecessary and even disadvantageous, since it removes the small amount of ethanol added to analytical-grade chloroform to prevent decomposition. This finding has since been confirmed by Ceriotti (private communication). Some other sources of interference with the reaction have been noted. Ceriotti[39] observed that TCA, but not PCA, inhibits the reaction of indole with DNA, but Keck[277] could not confirm this and even found an increased range of linearity of the Ceriotti reaction when TCA was used to extract the DNA. Waterlow and Weisz[216] preferred TCA as an extractant for liver nucleic acids, on the grounds that PCA caused a decrease in the intensity of colour formed with indole, especially when the tissue extracts made with PCA were stored for any length of time. Park and Ullrich[278] have noted that ammonium formate gives with indole an orange-pink colour not readily extractable with chloroform.

Hutchison, Downie and Munro[30] have applied the Ceriotti method directly to the DNA fraction of the Schmidt - Thannhauser procedure. They found that the values obtained from liver and spleen specimens were in good agreement with the phosphorus content of the DNA fraction. Similar values were obtained when the Ceriotti procedure was applied directly to the alkaline digest without separation of the DNA fraction. No colour was obtained when the procedure was applied to samples of liver cell cytoplasm devoid of DNA. This indicates that protein, the other constituent of the DNA fraction by the Schmidt - Thannhauser procedure, does not interfere with the Ceriotti determination; Ceriotti[133] observed no colour when casein hydrolysate was allowed to react with indole. Deken-Grenson and Deken[217] applied both the Ceriotti and diphenylamine procedures to hot PCA extracts of the DNA fraction prepared from plant, animal or bacterial sources. Excellent agreement between the two procedures was observed, although some of the deoxyribose had been destroyed by using 5 per cent. PCA at 90° C to make the extracts. Unspecified interfering substances have been encountered in hot sodium chloride extracts of beet petiole.[279]

Several authors[180,226,271,280,281,282,283] have studied the chemical reactions occurring between DNA or deoxyribosides and cysteine in sulphuric acid. Two distinct methods of DNA determination have emerged. In one procedure,[226] cysteine and DNA are allowed to react in the presence of concentrated sulphuric acid. This method can be used to cover the range 50 to 500 μg of DNA or 10 to 100 μg by Brody's[227] modification. This reagent produces coloured products with a variety of carbohydrates, but protein does not interfere.[180] The second procedure, involving the use of 75 per cent. sulphuric acid,[280] has been adapted by Stumpf[225] for assaying DNA in the range 25 to 550 μg. Amino acids, proteins and most carbohydrates do not interfere,[180] and this is presumably why Juni, Kamen, Reiner and Spiegelman[138] were able to apply this reaction to the DNA fraction of an alkaline digest of yeast that could not be examined by the diphenylamine reaction on account of interference.

Other methods for measuring deoxyribose have been applied to determining DNA. Webb and Levy[15] used the relatively specific reaction between deoxyribose and *p*-nitrophenylhydrazine to measure DNA over the range 10 to 300 μg. It has not apparently been used by other investigators. A highly sensitive and specific fluorimetric procedure capable of measuring 0·002 μg of DNA has been described by Kissane and Robins[284]; it depends on the fluorescence of the compound formed when 3,5-diaminobenzoic acid reacts with deoxyribose. Cohen[272] has described a procedure involving reaction of DNA with tryptophan in perchloric acid; this procedure is rather insensitive and is subject to interference by various carbohydrates. In this method, the pigment formed with DNA is extracted into isopentyl alcohol to separate it from the colour formed with protein, but Asimov *et al.*[271] observed some protein interference even when this extraction was carried out. von Euler and Hahn[218] describe conditions under which phloroglucinol reacts with DNA to give a purple colour that can be used for its assay. Waravdekar and Saslaw[285] propose a method in which deoxyribose is first made to react with periodate to give malonaldehyde, which produces a colour with 2-thiobarbituric acid; the optical density is proportional to the deoxyribose content of the specimen up to 2·5 μg. Finally, the method of Gurin and Hood,[224] based on the carbazole reaction,[223] and the anthrone method of Gary and Klausmeier[232] lack specificity

for carbohydrates and do not appear to have been applied to DNA measurement in biological materials.

In conclusion, it would appear that Burton's[261] modification of the diphenylamine reaction and Ceriotti's[133] indole procedure offer the most suitable general methods of measuring DNA without serious interference. The latter procedure is the more tedious, on account of the three extractions with chloroform, but provides greater sensitivity. The other methods, notably the cysteine reaction involving 75 per cent. sulphuric acid, the method of Webb and Levy[15] and the fluorimetric procedure,[284] may possess advantages of specificity and sensitivity in special circumstances.

Determination by ultra-violet absorption—Just as with the RNA fraction, the purine and pyrimidine bases of the DNA fraction can be determined by their strong ultra-violet absorption. Directly measuring the ultra-violet absorption of the fraction dissolved in alkali will give erroneously high readings because of the presence of tissue protein, which also absorbs in the ultra-violet region. However, several authors[5,128,175] have reduced the error from this source by taking readings at two wavelengths. This procedure will not completely eliminate the absorption due to protein unless the wavelengths chosen give identical readings for protein and different readings for DNA, but the conditions for obtaining this do not appear to have been established. An alternative procedure is to extract the DNA fraction with hot TCA[17,136,253,286] or hot PCA[23,47,73,80,217,251,287] and read the ultra-violet absorption of the extract. Hot PCA is usually preferred for this to TCA because of the latter's ultra-violet absorption. McIntire and Sproull[79] extracted the DNA with 0·1 N sulphuric acid in 10 per cent. sodium chloride. Scott, Fraccastoro and Taft[73] studied the conditions for extracting DNA by hot PCA and could find none under which the DNA was removed quantitatively without the extraction also of some ultra-violet absorbing amino acid material, and they recommend conditions that are a compromise. Extraction of amino acids by too vigorous use of hot PCA was also noted by Gardella and Lichtler.[251] Minagawa and Strauss[80] and Tsanev and Markov[47] have sought to overcome this difficulty by taking readings at two wavelengths in the ultra-violet region; the latter group of authors chose their two wavelengths on the basis of peptide material isolated from the RNA fraction of the digest, but this may not be representative of the protein of the DNA fraction. On the whole, the errors of measuring the DNA content of TCA or PCA extracts by their ultra-violet absorption at 260 or 268 mμ cannot usually be large, since several authors[17,23,253,254] have reported reasonable agreement between this method and other procedures for determining DNA content.

Determination by quantitative measurement of individual bases—Like the RNA fraction, the DNA fraction of the alkaline digest can be digested with 72 per cent. PCA at 100° C for 1 hour[12] to obtain the free bases, which can then be separated chromatographically. Conditions for quantitative PCA hydrolysis of DNA have been explored by Emanuel and Chaikoff.[288] This has been used to measure the amount of DNA in liver[124] and in bacteria,[123] the latter group of investigators having been able to account for 95 per cent. of the phosphorus of the DNA fraction by recovery of the bases. Kleinschmidt and Manthey[35] have preferred to use trifluoroacetic acid[289] to obtain the free bases from the DNA fraction in their assay procedure, because it is more effective in liberating the bases and does not char the chromatography paper. Stuy[201] treated the DNA fraction of bacteria successively with 0·1 N hydrochloric acid, trifluoroacetic acid and PCA in order to isolate the bases for paper chromatography. The recovery of the bases was in good agreement with the amount of DNA indicated by the diphenylamine reaction. The use of phosphorus pentoxide for hydrolysing DNA to bases, proposed by Narurkar and Sahasrabudhe,[290] does not appear to have been applied to analysis of the DNA fraction from the Schmidt - Thannhauser procedure. A method based on chromatographic separation of thymidylic acid after treatment of the DNA fraction with N hydrochloric acid for 1 hour at 90° C has been proposed.[291] To obtain the deoxyribonucleotides, deoxyribonuclease and phosphodiesterase have been allowed to attack the DNA fraction,[137] though this procedure seems not to have been used as the basis of any quantitative method for DNA.

Finally, several methods of measuring thymine specifically have been applied to the DNA fraction from the Schmidt - Thannhauser procedure. Abrams[249,250] reports an isotope-dilution method by adding labelled adenine and guanine to the DNA fraction, which is then hydrolysed to yield the free purines; these are isolated by silver precipitation, and the dilution of the isotope is measured. Marshak and Marshak[71] describe a similar procedure in which ^{14}C-labelled thymine is added after digestion of the DNA fraction with 72 per cent.

PCA, the mixture being then purified by paper chromatography. Thymine can be determined on the DNA fraction by microbiological assay[292]; a recent form of assay can detect 0·001 to 0·1 μg of thymidine.[293] A high degree of sensitivity has also been obtained with a fluorimetric procedure based on opening of the thymine ring and condensation to give a fluorescent product[254]; the application of this assay to the DNA fraction of various tissues gave results similar to those obtained by diphenylamine, phosphorus or ultra-violet measurements.

All of these procedures for determining DNA by recovery of bases are technically more complicated than the commoner ones.

RECOMMENDATIONS FOR THE USE OF THE SCHMIDT - THANNHAUSER METHOD—

Difficulties can arise at every stage in the Schmidt - Thannhauser procedure for determining nucleic acids. The following recommendations may help to circumvent the major hazards.

(*a*) Preliminary treatment: it is essential to avoid degrading the nucleic acids during the preliminary removal of acid-soluble compounds and subsequent extraction of lipids. Degradation can occur through exposure to high degrees of acidity or, at lower levels of acidity, through a rise in temperature. For this reason, TCA is a safer protein precipitant than PCA for removal of acid-soluble tissue constituents. Similarly, if heat is used during extraction of lipids from the tissue, degradation of DNA can occur because of acid carried over into the lipid solvents. This can be prevented by buffering the first lipid solvent (usually ethanol) with sodium acetate[56] or alternatively by using lipid solvents without heat. If precautions against degradation of DNA are not taken, apurinic acid may form and subsequently appear in the RNA fraction after alkaline digestion.

(*b*) Alkaline digestion: there appears to be no justification for using the 15-hour period of incubation with N alkali originally recommended by Schmidt and Thannhauser.[9] A 1-hour period of digestion in N alkali at 22° C[73] or in 0·3 N alkali at 37° C[38] has been successfully used on various tissues for extracting RNA in a form no longer precipitable on acidification of the digest. The 1-hour period of alkaline digestion has the advantage over longer periods of digestion that little of the tissue protein becomes acid-soluble, and thus the RNA contained in the acid-soluble fraction of the digest can be accurately determined by ultra-violet absorption. The adequacy of the 1-hour period of digestion in liberating all the RNA from the tissue can be verified by carrying out orcinol and phosphorus measurements on the RNA fraction and phosphorus determinations on the DNA fraction after different periods of alkaline digestion.

It is also wise to ensure that the DNA has been fully precipitated by acidification at the end of incubation. This can be done by testing the RNA (acid-soluble) fraction for DNA, for example by applying the Ceriotti[133] indole procedure for deoxypentose, which is highly sensitive. If any deoxypentose is detected in the RNA fraction, then the earlier stages in the determination should be reviewed for possible degradation of DNA to apurinic acid.

Finally, it is unwise to ignore portions of tissue that resist digestion in alkali. These should be separated from the digest and their significance assessed by determining the amount of phosphorus and deoxypentose directly on the sediment and by making a hot PCA extract on which ultra-violet absorption can be examined at several wavelengths.

(*c*) Determination of RNA: on account of the presence of appreciable amounts of non-RNA phosphorus in the acid-soluble fraction of most tissues, determination of phosphorus on this fraction will give values commonly 15 to 20 per cent. more than the RNA phosphorus content and sometimes considerably higher. An adequate estimate of the amount of RNA present can sometimes be obtained by ribose determination, but a convenient procedure with a considerable degree of precision is to measure ultra-violet absorption at 260 mμ after a 1-hour period of digestion in 0·3 N alkali at 37° C or N alkali at 22° C. A simple protein determination (*e.g.*, Lowry *et al.*[294]) will demonstrate whether the amount of protein present in the RNA fraction is small enough to be ignored as a source of ultra-violet absorption; Fleck and Munro[38] separated the protein-degradation products from the acid-soluble fraction and found that 1 μg per ml contributed about 0·0008 to the optical density at 260 mμ. The purity of the RNA fraction for ultra-violet absorption measurements can be further checked by examining the ultra-violet spectrum and comparing it with the spectrum of RNA, preferably of the RNA isolated from the tissue under consideration. If these procedures still leave

doubts about the accuracy of the ultra-violet absorption measurements for determining RNA, it may be necessary to isolate the individual bases or nucleotides from the acid-soluble fraction. This can be done with least peptide contamination by making the 1-hour acid-soluble fraction alkaline again and continuing digestion for 18 hours, the mononucleotides then being isolated by ionophoresis and the individual nucleotides determined by phosphorus recovery. Finally, it is prudent to make certain that the RNA has been fully extracted from the tissue by the 1-hour period of alkaline digestion. This can conveniently be done by digesting samples of the tissue for 18 hours and then determining the phosphorus and pentose in the acid-soluble fraction. If the values are similar to those obtained after 1 hour's digestion in alkali, it is unlikely that further RNA has been released by prolonging the period of alkaline digestion.

(*d*) Determination of DNA: for this, determinations of phosphorus and deoxypentose will usually give reliable figures for the amount of DNA present. For the determination of deoxypentose, Burton's[261] modification of the diphenylamine reaction and Ceriotti's[133] indole procedure are satisfactory and relatively simple to carry out; Burton's procedure is technically easier, but less sensitive. The determinations should be performed directly on the DNA fraction dissolved in alkali. The practice of extracting the DNA fraction with hot TCA or PCA (Schneider's[171] modification of the Schmidt - Thannhauser procedure) can lead to somewhat low values for DNA; ultra-violet measurements on the extract are subject to errors resulting from extraction of aromatic amino acids.

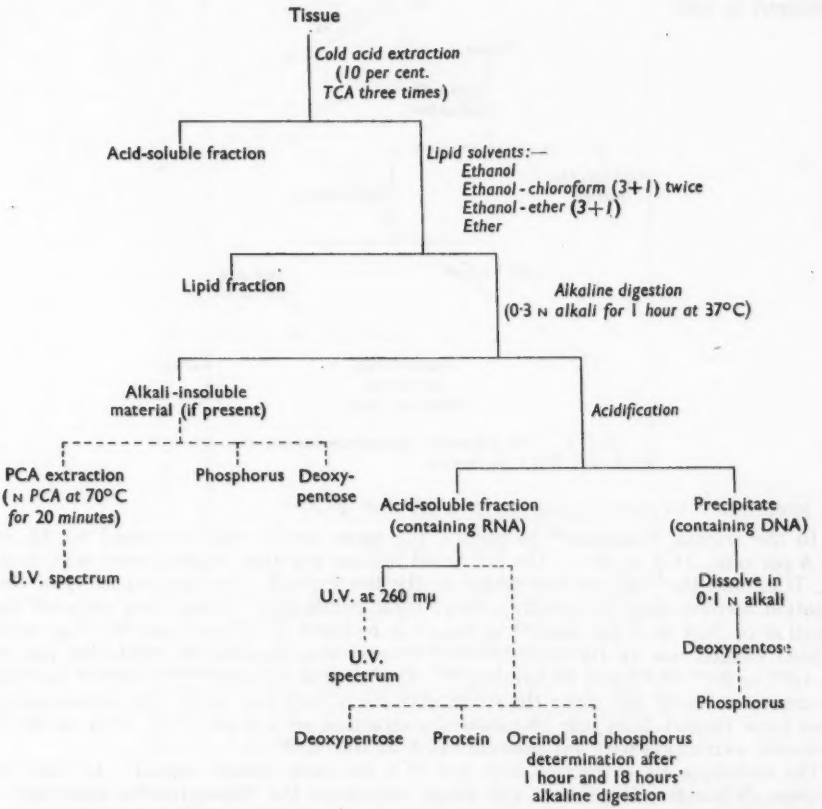
Tissue
Cold acid extraction (10 per cent. TCA three times)
Acid-soluble fraction
Lipid solvents:— Ethanol Ethanol - chloroform (3+1) twice Ethanol - ether (3+1) Ether
Lipid fraction
Alkaline digestion (0·3 N alkali for 1 hour at 37°C)
Alkali-insoluble material (if present)
Acidification
PCA extraction (N PCA at 70°C for 20 minutes)
Phosphorus
Deoxy-pentose
Acid-soluble fraction (containing RNA)
Precipitate (containing DNA)
U.V. spectrum
U.V. at 260 mμ
Dissolve in 0·1 N alkali
U.V. spectrum
Deoxypentose
Phosphorus
Deoxypentose
Protein
Orcinol and phosphorus determination after 1 hour and 18 hours' alkaline digestion

Fig. 4. A modified form of the Schmidt - Thannhauser procedure (full lines represent routine method; broken lines represent recommended check procedures to be carried out initially)

In order to verify that there are no substances in the DNA fraction interfering with deoxypentose or phosphorus determination of DNA, it is sometimes convenient, for tissues in which nuclei can be separated from the cytoplasm, to prepare the "DNA" fraction from the cytoplasm and check that it contains no phosphorus and does not react positively with deoxypentose reagents.[30]

These recommended modifications of the Schmidt - Thannhauser procedure are shown schematically in Fig. 4.

2. The Schneider procedure

In Schneider's[10] original procedure for nucleic acid (Fig. 5), the tissue is first extracted with cold acid and with lipid solvents as in the Schmidt - Thannhauser procedure, but thereafter the nucleic acids are simultaneously extracted from the tissue with 5 per cent. TCA at 90° C. Schneider used the orcinol reaction to measure the amount of RNA in the extract and the diphenylamine or carbazole reaction to measure the DNA content. In a later modification, Schneider, Hogeboom and Ross[29] used PCA at 90° C to extract the nucleic acids.

Many authors have applied either the TCA or the PCA procedure to nucleic acid determination in various biological materials. In so doing, the conditions of extraction originally proposed by Schneider have frequently been varied. In addition, some investigators have measured the total extracted nucleic acids by the ultra-violet absorption of the extract; in order to obtain the individual amounts of RNA and DNA present, various methods have been applied to the extract. Each of these aspects of the Schneider procedure will now be considered in turn.

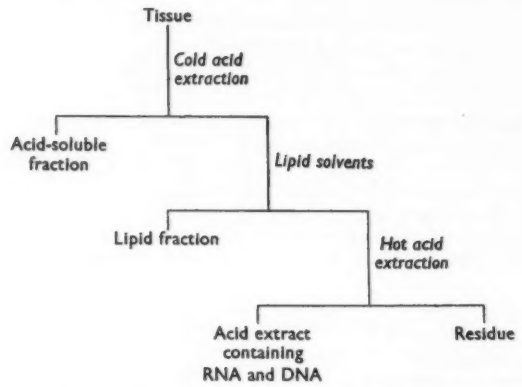

Fig. 5. The Schneider procedure for determination of RNA and DNA in tissues

The efficiency of extraction with TCA and PCA—

In the original Schneider[10] procedure, the tissue residue was extracted for 15 minutes with 5 per cent. TCA at 90° C; the extracted residue was then washed once with cold 5 per cent. TCA, and the washing was added to the hot extract. Subsequent users of the TCA extraction method have frequently altered these conditions. Some have reduced the concentration of TCA to 3 per cent.[295] or raised it to 5·9[296] or 7·5 per cent.[197] The extraction has been carried out at 100° C,[124,196,197,297,298] and the duration of extraction has ranged from 10[295] to 20[197,299,300] and 30 minutes.[196] Also, several authors[300,301,302,303,304] have thought it necessary to carry out more than one extraction with hot TCA. In consequence, conditions have ranged from one 10 minutes' extraction with 3 per cent. TCA at 90° C[295] to 20 minutes' extraction with 7·5 per cent. TCA at 100° C.[197]

The technique for extraction with hot PCA has been equally varied. In their original procedure, Schneider, Hogeboom and Ross[29] extracted the tissue residue once with 6 per cent. (1 N) PCA at 90° C for 15 minutes. The concentration of PCA used by some other workers has varied from 3 per cent. (0·5 N)[75,261,305,306,307,308,309,310] to 5 (0·8 N),[217,311] 9 (1·5 N)[312] and even 10 per cent. (1·6 N)[234,302,313,314] Many users of the procedure have reduced

the temperature of extraction to 70° C,[75,261,305,307,308,310,315,316] but 80° C[234,302,312,313,314] and 95° C[306] have also been used. The time of extraction has commonly been extended to 20 minutes,[75,305,307,309,310,315,316] but 30[234] and 50 minutes[308] have also been used. Finally, many authors[75,261,302,305,306,307,310,312,313,314,315,317] have carried out more than one extraction with hot PCA.

Insufficient work has been done to determine whether these variations in the conditions of extraction are important. In his original publication, Schneider[10] examined the extraction of RNA and DNA from specimens of liver with 5 per cent. TCA at 90° C and concluded that periods over 10 minutes were sufficient for maximum extraction. When he varied the concentration of TCA from 0 to 10 per cent., he found that concentrations of 3·4 per cent. upwards gave maximum yields. Hutchison, Downie and Munro[30] studied the efficiency of extracting RNA and DNA with hot PCA from liver and spleen. They used two temperatures that have been commonly employed for PCA extraction, the original 90° C of Schneider, Hogeboom and Ross[29] and also 70° C[75,261,305,307,308,310,315,316] and measured the extracted RNA by the orcinol reaction[202] and DNA by Ceriotti's indole reaction.[133] The results obtained on specimens of rat liver are shown in Fig. 6 as percentages of the RNA and DNA values found when the same sugar reactions were applied to the RNA and DNA fractions prepared by the Schmidt - Thannhauser method. Similar results were obtained

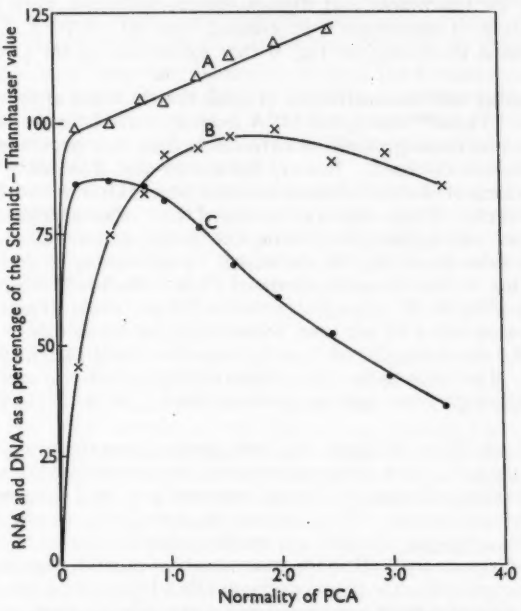

Fig. 6. Effect of variation in concentration of PCA used for extracting nucleic acids from rat liver tissue residues at 70° and 90° C: curve A, RNA (at 70° C); curve B, DNA (at 70° C); curve C, DNA (at 90° C). Two extractions each of 20 minutes' duration were carried out at every concentration of PCA, and the orcinol and Ceriotti reactions, respectively, were used for determining RNA and DNA in the extract. The values obtained are plotted as percentages of the value obtained by the Schmidt - Thannhauser method. (Modified from Hutchison, Downie and Munro[30])

with specimens of rat spleen. At both temperatures of extraction, the RNA was fully removed from the tissue at low concentrations of PCA, and further increments in acid concentration led to the appearance of additional small amounts of orcinol-reacting material. On the other hand, the apparent DNA content of the tissue extracts prepared at 90° C reached

a maximum value with 0·5 N PCA and thereafter diminished rapidly with increasing concentrations of PCA; at all concentrations of PCA, less DNA was found in the 90° C extracts than by applying the same colour reaction to the DNA fraction from the Schmidt - Thannhauser procedure. When the tissue was extracted with PCA at 70° C, the DNA content found was close to the value obtained by the Schmidt - Thannhauser procedure between 1 and 2 N PCA, but diminished at higher concentrations. With 0·5 N PCA, which has been frequently used to extract tissues at 70° C,[75,305,307,310] the amount of DNA obtained was 25 per cent. lower than the value by the Schmidt - Thannhauser method.

Scrutiny of the published literature shows that several other workers have found the conditions for extracting DNA with hot acid to be critical. Chiba and Sugahara[318] studied extraction of nucleic acids from plant particles by heating in PCA at 70° C for 30 minutes and observed that, although 3 per cent. (0·5 N) PCA was adequate for removal of the RNA, 6 per cent. (1 N) PCA was needed for extracting the DNA. In contrast, Burton[261] examined the efficiency of extraction of DNA from bacteria by PCA at 70° C and found that two 20-minute extractions with 3 per cent. (0·5 N) PCA removed 96 per cent. of the total DNA that could be obtained by four extractions, whereas with 6 per cent. (1 N) PCA the yield was reduced to 86 per cent. The effect of temperature on DNA destruction shown in Fig. 6 is confirmed by the observations of Deken-Grenson and Deken,[217] quoted in an addendum to their paper. When samples of DNA were heated in 5 per cent. (0·8 N) PCA for 15 minutes, the values obtained by the indole and diphenylamine reactions were increased by 10 per cent. if the temperature of extraction was reduced from 90° to 70° C. It is apparent from these findings and those illustrated in Fig. 6 that extraction at 90° C with PCA inevitably leads to low results.

Besides temperature and concentration of acid, the duration of extraction can influence the result. Hirtz and Fayet[304] extracted DNA from epithelial tissues with 5 per cent. TCA at 90° C and obtained increasing yields as extraction time was prolonged up to 60 minutes, but thereafter the values declined. Bakay, Kirschner and Toennies[281] observed a similar effect when preparations of deoxyribonucleoprotein were extracted with TCA at 100° C for increasing lengths of time. These authors also found that, when specimens of RNA and DNA were heated in TCA for varying lengths of time, the deoxypentose values diminished steadily, whereas the ribose values were slightly increased. Destruction of deoxyribose also occurs with continued heating in low concentrations of PCA. Burton[261] heated samples of DNA in 3 per cent. (0·5 N) PCA at 70° C and observed a 7·5 per cent. diminution in deoxyribose content after 30 minutes and a 14 per cent. reduction after 60 minutes. Heating with 5 per cent. TCA at 90° C for these lengths of time had similar though slightly less marked effects on colour formation. The more facile degradation of deoxyribose by acid has also been noted by investigators preparing ribose and deoxyribose from nucleic acids for chromatographic isolation.[319]

It is apparent from these findings that two factors operate when hot acid is used to extract DNA from tissues: at low temperatures and low concentrations of acid, DNA may be incompletely extracted, whereas at higher temperatures and concentrations, significant destruction of deoxyribose occurs. These factors do not appear to affect the determination of RNA by ribose measurements; the RNA is readily extracted from the tissue, and the sugar remains stable to acid treatment. Besides concentration of acid, temperature and duration of extraction, other factors influence the recovery of DNA by hot acid extraction. Hutchison, Downie and Munro[30] observed that tissues differed widely in the extractability of their DNA by 0·5 N PCA at 70° C. Lindigkeit and Rapoport[320] claimed that the extraction of DNA from liver with hot TCA could be reduced by addition of red cells to the tissue, but Hutchison, Downie and Munro[30] were unable to confirm this observation with added blood. Hermann and Speck[321] obtained lower recoveries of DNA from tissues fixed in chromate, and it has been observed[322,323,324] that the extractability of nucleic acids from histological sections by means of hydrochloric acid, PCA or TCA depends on the nature of the tissue and the histological fixative as well as on conditions of acidity and the temperature of extraction. It is thus apparent that the conditions for optimum extraction of nucleic acids from tissues will vary with the nature and treatment of the material and cannot be predicted.

DETERMINATION OF NUCLEIC ACIDS IN THE EXTRACT—

The total nucleic acid content of the extract has been assessed by phosphorus determination[10] and by copper precipitation of purine bases,[325] but most commonly by ultra-violet

absorption measurements.[17,31,216,326,327,328] It has been claimed that ultra-violet methods can be used even with TCA extracts, in spite of the known ultra-violet absorption of this extractant. Di Carlo and Schultz[326] and Waterlow and Weisz[216] state that, in their procedures, the TCA is so diluted that it has no significant absorption at 260 mμ. Logan, Mannell and Rossiter[17] used a suitable TCA blank; Bieber, Spence and Hitchings[328] extracted the TCA with ether before making ultra-violet measurements. When the total nucleic acid content of the acid extract is measured by ultra-violet absorption, it is necessary to treat the standard in the same manner, since both nucleic acids show an increment in absorption at 260 mμ (hyperchromic effect) when heated in acid.[17,236,329] It should also be remembered that protein-degradation products extracted by the hot acid can contribute to the ultra-violet absorption of the extract. Schneider[10,330] himself noted that the nitrogen content of TCA tissue extracts was consistently higher than their nucleic acid content indicated, and extraction of protein-degradation products has been noted by authors who used hot TCA[234,268,331] or hot PCA[234,332] as the extractant. Logan, Mannell and Rossiter[17] did not obtain evidence of protein-degradation products in the TCA extracts of nervous tissue until heating had been prolonged. On the other hand, considerable amounts of ultra-violet absorbing protein break-down products were extracted when 6 per cent. (1 N) PCA was substituted for 5 per cent. TCA.

For the determination of individual amounts of RNA and DNA in the extract, various colorimetric reactions have been used, chiefly orcinol for RNA and diphenylamine or indole for DNA. The merits of these individual reactions have already been discussed, and here it is only necessary to indicate sources of interference peculiar to the Schneider method.

In his original procedure, Schneider[10] pointed out that DNA reacts with orcinol and that in consequence a correction has to be applied to the determinations with orcinol in order to obtain the true RNA value of the extract. Schneider found that DNA gave 12 per cent. of the colour formed by the same weight of RNA, and this was also observed by von Euler and Hahn,[210] Price[301] and Hutchison, Downie and Munro.[30] Paigen and Kaufmann[124] obtained a figure of 19 per cent. for liver DNA, but Steele *et al.*[259] obtained only 2·5 per cent. by their micro method, and Smillie and Krotkov[32] obtained a colour equivalent to only 0·3 per cent. of that of RNA when they used Markham's modification of the orcinol procedure. The presence of materials other than DNA in the hot-acid extract can also lead to errors with the orcinol reaction. With TCA extracts of human liver, Waterlow and Weisz[216] obtained erroneously high orcinol values because of extracted glycogen. Orcinol-reacting contaminants have been observed in TCA extracts of bacteria[197] and in PCA extracts of Chlorella.[32]. In a survey of animal and plant tissues and bacteria, Deken-Grenson and Deken[217] found that the orcinol values obtained on PCA extracts tended to be erroneously high, especially for plants. These high values were presumably due to carbohydrates other than ribose in the extract.

Measurement of the DNA content of the extract has usually been carried out by the diphenylamine reaction, though the carbazole and indole procedures are also used. Substances that interfere with the diphenylamine reaction have been frequently encountered in hot acid extracts of tissues. Only a few of these have been identified. Hot TCA extracts of amphibian eggs were found by Finamore[333] to contain a substance, apparently an octopeptide, that increased the colour produced in the diphenylamine reaction. With certain plant materials, the diphenylamine reaction applied to hot PCA extracts has been found to give erroneously high results unless preliminary extraction of the tissue with light petroleum was carried out.[334] On the other hand, Newton and Wildy[310] found that the intensity of the diphenylamine reaction with PCA extracts of tissue culture cells was not dependent on prior removal of lipids. The colour given by diphenylamine with hot TCA extracts of serum[335,336,337] and of bovine tonsil[336] is apparently due to release of mannose from glycoproteins present in the original extract; reaction with glycoproteins has long been recognised as a source of error in the diphenylamine reaction.[338] On most other occasions the identity of the interfering material has not been ascertained. Potter, Recknagel and Hurlbert[339] noted that PCA extracts of tissues contained unidentified substances that gave spurious colours with diphenylamine, and Schneider[181] confirmed this on extracts made from liver samples. Deken-Grenson and Deken[217] found that PCA extracts of various plant and animal materials gave higher DNA values by the diphenylamine than by the indole reaction. Logan, Mannell and Rossiter[17] obtained erroneously high readings for DNA when the diphenylamine reaction was applied to hot TCA extracts of nervous tissue, although this method gave satisfactory values when used on the DNA fraction prepared from the same tissue by the

Schmidt - Thannhauser procedure. Substances interfering with the diphenylamine reaction for DNA have also been observed in hot acid extracts of bacteria[197,253] and of fungi.[80]

The indole reaction[133] can also be used to measure the DNA content of the extract; Ceriotti[39] observed that TCA, but not PCA, inhibited the reaction with DNA. On the other hand, Waterlow and Weisz[216] found TCA to be without effect; they found, however, that the intensity of colour formation decreased when tissue extracts made with PCA were stored for any length of time, presumably owing to destruction of deoxyribose. This effect of storage in PCA on DNA was also observed by Deken-Grenson and Deken.[217] These authors examined the DNA content of various tissues by the Schneider PCA, the Schmidt - Thannhauser and the Ogur - Rosen methods of extraction. Good agreement was obtained between the Schneider and Schmidt - Thannhauser procedures when the indole reaction was used for DNA determination, but the diphenylamine method gave high values when applied to the Schneider PCA extract. Unfortunately their extraction procedure was subsequently found to be unreliable (see addendum to their paper). Application of the Ceriotti procedure to hot acid extracts of ova[188,328,340] has led to higher values for DNA content than those obtained by isotope dilution of thymine or microbiological assay.

Finally, to counterbalance these occasions when the assay of RNA or DNA in hot acid extracts was found to be in error, there are many records in the literature of investigators who obtained reasonable agreement between the Schneider and some other method of nucleic acid determination, generally that of Schmidt - Thannhauser. Schneider[171] himself compared his method with a slightly modified Schmidt - Thannhauser procedure and found satisfactory agreement for a range of mammalian tissues. Agreement between the two methods has also been claimed for liver,[31,341,342] brain,[342] reticulocytes,[343] calf thymus,[344] ascites tumour cells,[345] isolated nuclei,[128] leaves[37] and bacteria.[253,327] For placenta, Schneider's method failed to agree with that of Schmidt - Thannhauser, but gave values similar to those obtained by Hammarsten's method.[299,346]

RECOMMENDATIONS FOR THE USE OF THE SCHNEIDER PROCEDURE—

The Schneider procedure has proved attractive to many workers because the results are more rapidly obtained than are those by the Schmidt - Thannhauser method and because fewer manipulations are involved. The former advantage is probably no longer valid if the short period of alkaline digestion recommended earlier here is used in the Schmidt - Thannhauser procedure. Against the relative simplicity of the Schneider procedure must be placed the sources of error listed in detail above.

First, there is now irresistible evidence that the Schneider extraction procedure can result in both incomplete extraction of DNA and destruction of deoxyribose. From Fig. 6 it can be seen that conditions can be chosen under which extraction is relatively complete and destruction is minimal when PCA at 70° C is the extractant, but scrutiny of the literature has shown that the conditions required for complete recovery of DNA vary from one tissue to another, so that general recommendations about hot acid extraction cannot be given with confidence. Thus Hutchison, Downie and Munro[30] observed in one of their experiments (see Fig. 6) that 25 per cent. of the DNA of liver remained in the tissue after two 20-minute treatments with 3 per cent. (0·5 N) PCA at 70° C, whereas only 3 per cent. of the total DNA was not recovered in the extract when 10 per cent. (1·6 N) PCA was used. By contrast, Burton[261] obtained 96 per cent. of the total extractable DNA by treating bacteria twice with 3 per cent. (0·5 N) PCA at 70° C for 20 minutes, but only 86 per cent. when 6 per cent. (1 N) PCA was used as extractant. It is thus a matter for the analyst to establish, by comparison with some other procedure, such as the Schmidt - Thannhauser method, that his conditions for extraction provide adequate recovery of DNA. In this connection, it is apparent (Fig. 6) that with PCA as the extractant it is best to keep the temperature of extraction low.

Secondly, sources of error in determining the nucleic acids present in the extract have been observed rather frequently. The total nucleic acid content is conveniently determined by ultra-violet absorption, provided that the extract does not contain significant amounts of protein-degradation products. It is best to reassure oneself on this point by examining the ultra-violet spectrum of the extract to see whether absorption due to the presence of protein can be detected; a test[294] for protein and its degradation products can also be carried out. The determination of RNA in the extract by the orcinol procedure has sometimes given erroneously high values. This may be avoided[31] by determining the total nucleic acid content of the extract by ultra-violet absorption and subtracting the value found

for its DNA content by a colorimetric procedure. Several investigators have observed that measurement of the DNA content of the extract by the diphenylamine reaction gives too high values for some materials. On the basis of results obtained by Deken-Grenson and Deken[217] for a variety of materials, it is possible that Ceriotti's indole procedure may provide more reliable measurements of the DNA content of hot acid extracts.

3. The procedure of Ogur and Rosen

This method was originally proposed by Ogur and Rosen in 1949[347] for determining small amounts of nucleic acids in plant materials. The principle of the technique depends on preferential solubilisation of RNA by cold PCA and extraction of DNA with hot PCA. The nucleic acid present in each extract is then measured by its ultra-violet absorption. As fully described[16] for use on plant tissues, the procedure (see Fig. 7) consists in first extracting the tissue with lipid solvents, the acid-soluble small molecules being then removed by brief treatment with cold 1 per cent. (0·2 N) PCA. The residue is exposed to 6 per cent. (1 N) PCA for 18 hours at 4° C, a treatment claimed to take out all the RNA. The DNA is subsequently removed by two 20-minute extractions at 70° C with 3 per cent. (0·5 N) PCA. For animal tissues Ogur and Rosen[16] recommend extraction with 6 per cent. (1 N) PCA at 80° C for two 30-minute periods in order to ensure complete removal of DNA.

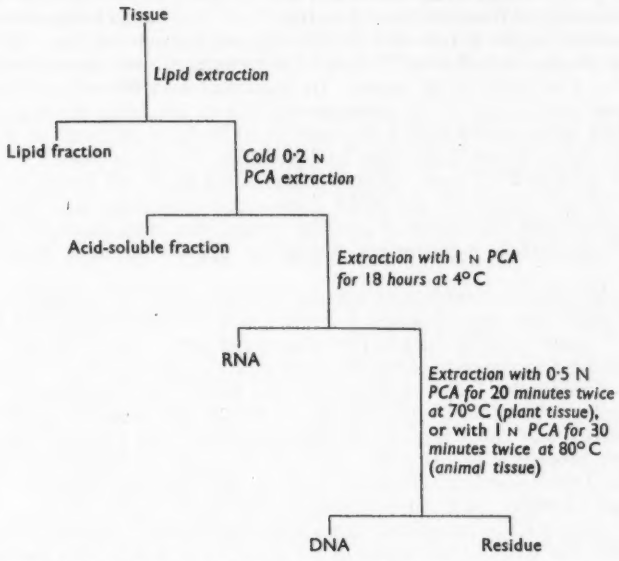
Tissue
Lipid extraction
Lipid fraction
Cold 0·2 N PCA extraction
Acid-soluble fraction
Extraction with 1 N PCA for 18 hours at 4°C
RNA
Extraction with 0·5 N PCA for 20 minutes twice at 70° C (plant tissue), or with 1 N PCA for 30 minutes twice at 80° C (animal tissue)
DNA
Residue

Fig. 7. The Ogur - Rosen procedure for determination of RNA and DNA in tissues

Ogur and Rosen[16] examined the adequacy of their procedure for separating the two nucleic acids in samples of rabbit liver. Ultra-violet measurement of the amount of RNA extracted by cold PCA and of DNA removed by hot PCA were in excellent agreement with the amounts obtained by applying the orcinol and diphenylamine reactions, respectively, to the same extracts. The values also agreed with those for the liver specimens by Schneider's TCA extraction procedure. In a later study of plant buds, in which 10 per cent. (1·6 N) PCA was used for 18 hours at 4° C to extract the RNA and 10 per cent. PCA at 80° C for 20 minutes to remove the DNA, Ogur and his colleagues[334] again observed good agreement between the ultra-violet absorption of the DNA extract and its content of DNA measured by the diphenylamine reaction. Satisfactory results were also obtained by Holmes, Mee, Hornsey and Gray[348] when they compared the Ogur - Rosen and Schmidt - Thannhauser procedures in the analysis of plant root tips.

Few other investigators have found the Ogur - Rosen procedure to be satisfactory for quantitative separation of RNA from DNA. Two sources of error have been encountered: first, a number of workers have observed that the cold PCA extraction is inadequate for removing all the RNA from the material; conversely, a few investigators have observed that part of the DNA is extracted into the cold PCA along with the RNA. The evidence for each of these will now be considered.

Many authors have found that RNA is not completely extracted by cold PCA under the conditions laid down by Ogur and Rosen, and in consequence this unextracted RNA appears in the DNA fraction as ultra-violet absorbing material. This difficulty has been repeatedly encountered in analysis of plant material. Exposure of tobacco-leaf preparations to cold PCA for 18 hours was not effective in removing the RNA completely from the material,[215] a finding confirmed by identifying uracil in the DNA fraction.[349] In a study of the conditions suitable for extracting RNA from tobacco-leaf fibre and chloroplasts, Holden[350] failed to achieve complete removal of RNA by treatment with 6 per cent. (1 N) PCA for 18 hours at 4° C, although most of the RNA could be removed by raising the temperature of extraction to 16° C. With pea seedling roots,[75] exposure to cold 6 per cent. (1 N) PCA for 30 hours was found to leave considerable amounts of unextracted RNA in the residue, and Ts'o and Sato[351] noted that 72 hours' treatment with cold 6 per cent. (1 N) PCA was necessary for removing all the RNA from certain cell fractions isolated from pea seedlings. Failure to extract all the RNA from pea seedlings in 20 hours was also suspected by Stafford,[214] who found more ultra-violet absorbing material in the subsequent hot PCA extract than could be accounted for by the amounts of DNA revealed by the diphenylamine reaction. From microsomes of beet petioles Martin and Morton[279] were able to extract only about half the RNA with cold 6 per cent. (1 N) PCA in 30 hours. In Euglena, Soldo[352] and Smillie and Krotkov[32] observed the presence of significant amounts of RNA in the DNA fraction after samples of the organism had been treated with 6 per cent. (1 N) PCA for 24 hours at 4° C. Difficulties in applying the Ogur - Rosen procedure to Neurospora[80] and to yeast[217,353] have also been reported. Two workers[253,354] have observed that some 40 to 44 hours' contact with cold PCA were needed to extract all the RNA from certain bacteria, and Deken-Grenson and Deken[217] also noted that the standard 18-hour period was inadequate to remove the RNA completely from *Bacterium megatherium*, though it appears to have been successful with *Bacillus subtilis*.

Similar evidence for incomplete extraction of RNA under the conditions recommended by Ogur and Rosen[16] has been obtained in several well documented studies made on animal tissues. Moulé[23] observed with chicken liver that 6 per cent. (1 N) PCA removed only 64 per cent. of the total RNA in the tissue after 18 hours at 4° C and some 78 per cent. after 36 hours of contact; the unextracted RNA appeared in the DNA fraction removed with hot PCA. Greater ultra-violet absorption by the hot PCA extract than could be accounted for by its deoxyribose content was also observed by Deken-Grenson and Deken[217] when they applied the Ogur - Rosen procedure to rat liver. Cold PCA is also ineffective in removing all the RNA from isolated cytoplasmic fractions of the liver cell[355]; the remaining RNA was extracted with hot PCA and identified by sugar reactions and by isolation of its bases. The inadequacy of cold PCA to extract RNA completely from liver preparations explains why Badolato and Calabrese[125] found the RNA - DNA ratio of rat liver to be 1·3 by this method of analysis, whereas other methods of analysis give a ratio of about 3; Hadjiolov[356] also noted that analysis of rat liver by the Ogur - Rosen procedure indicated a smaller RNA content and a larger DNA content than those recorded by other authors using different analytical procedures. On the other hand, Seifter, Muntwyler and Harkness[314] observed good agreement between RNA determinations made on liver samples by the Ogur - Rosen and the Schneider TCA methods, and Rose and Schweigert[357] claim satisfactory extraction of RNA from spleen, liver and kidney by a 24-hour period of treatment with cold 9 per cent. (1·5 N) PCA. The Ogur - Rosen procedure has proved inadequate to extract all the RNA from embryos and hens' eggs in the hands of two independent investigators,[358,359] who were able to identify the unextracted RNA in the DNA fraction. A considerable part of the RNA of pig-heart sarcosomes and of mammary gland remains unextracted after two 18-hour periods in cold PCA,[360] and it has been noted[302] that up to 11 per cent. of the RNA in the salivary gland of Drosophila larvae resists extraction with cold PCA under the standard conditions for the Ogur - Rosen procedure.

The second criticism of the Ogur - Rosen technique is that some of the DNA in the sample may be solubilised by the cold PCA used in the extraction of RNA. Some authors

have tested for the presence of deoxyribose in the cold PCA extract. Negative results were obtained for bacteria,[253] Euglena[32] and lymphoid cells.[361] Rose and Schweigert[357] also obtained no diphenylamine-positive material when cold 9 per cent. (1·5 N) PCA was used to extract specimens of liver and kidney, but they were unable to apply this extractant to spleen on account of the loss of DNA into the cold extract. Loss of deoxyribose-reacting material from chick embryo samples into cold PCA was found to represent only 3 per cent. of the total DNA of the tissue.[362] Since the initial effect of acid on DNA is to cause loss of purine bases,[131,132] examination of the cold PCA extract for deoxyribose may be a relatively insensitive method of detecting DNA break-down. Heyes[75] has used a chromatographic procedure to examine loss of bases when DNA isolated from pea seedling roots is exposed to cold 6 per cent. (1 N) PCA for 18 hours; when the DNA was prepared from the apical region of the root, the cold acid treatment caused a loss of purine bases, whereas treatment of DNA derived from the basal region led to loss of pyrimidine and purine bases and also some phosphorus, indicating disintegration of the DNA chain. Other evidence suggestive of DNA break-down under the influence of cold PCA has been obtained. Loeb and Dickinson[363] found that cold PCA extracted more ultra-violet absorbing material from thymus and tumour cells than could be accounted for by the amount of phosphorus in the extract and concluded that some of the purine bases of DNA had been extracted by the cold acid. Bhargava, Bishop and Work[364] found more ultra-violet absorbing material in the cold PCA extract of spermatozoa than could be explained by the amount of ribose present and concluded that about 5 per cent. of the DNA had been extracted by cold PCA. Ogur and his colleagues[365] applied the Ogur - Rosen procedure to yeast and found that the values for DNA in this organism were 15 per cent. lower than those obtained by the Schneider PCA extraction technique; they attributed this to loss of DNA into the cold PCA fraction.

The frequently reported failure of cold PCA to extract RNA quantitatively has led a number of investigators to raise the temperature of extraction. Holden[350] studied conditions for the extraction of RNA from tobacco leaf at different temperatures and could find no conditions of temperature or of PCA concentration under which RNA was extracted completely without solubilising some of the DNA. Steinert[359] extracted embryonic tissues with 5 per cent. (0·8 N) PCA for 20 hours at room temperature in order to achieve complete removal of RNA, but observed that the DNA of the tissue lost appreciable amounts of purine bases under these conditions. Considerable losses of DNA were observed by Olmsted and Villee[76] when they attempted to remove RNA from liver cell nuclei by overnight treatment with 1 N PCA at room temperature. On the other hand, Berenbom and Peters[366] claim that separation of RNA and DNA can be achieved by extraction at 25° C if the period of action of PCA is kept short; they used three 20-minute treatments with 1 N PCA at 25° C and found that less than 10 per cent. of the RNA in various rat tissues escaped extraction, whereas less than 3 per cent. of the tissue DNA was rendered soluble by the procedure. They supported this claim, based on ribose and deoxyribose determinations, by showing that thymine was absent from the RNA fraction and uracil from the DNA fraction subsequently extracted with hot PCA. The success of Berenbom and Peters's procedure is a little surprising, since Bonar and Duggan[33] have found that exposure of samples of thymus DNA to concentrations of PCA as low as 0·2 N at room temperature leads to solubilisation of 5 per cent. of the sample in 40 minutes.

The Ogur - Rosen procedure has been used successfully for differential extraction of RNA and DNA in the course of cytochemical studies on plants,[367] protozoa[368] and vertebrate tissues.[369] For other material the procedure has proved less effective. In a histochemical study of bacteria,[370] the extraction time had to be prolonged to 31 hours in order to remove all the RNA. Although Koenig and Stahlecker[371] found the Ogur - Rosen procedure satisfactory for histochemical studies on liver, extraction at a higher temperature was needed for complete removal of RNA from nerve cells. Under these conditions, there was little margin of safety between the time needed for extraction of RNA and that needed to remove DNA. This was also noted by Di Stefano,[372] who used 1 N PCA at 25° C to extract RNA from fixed sections of rat pancreas. All the tissue RNA was extracted by a 6-minute treatment with PCA, but after 9 minutes the DNA began to disintegrate. Edström[373] could not confirm that all the RNA was extracted in 6 minutes. The extractability of RNA from histological specimens is also influenced by the method of fixation.[374] Thus the use of PCA for histochemical purposes has led to the same conclusions as those derived from chemical

analysis of nucleic acids in tissues; extraction with PCA that has proved effective in one set of circumstances may not be applicable in others.

COMMENTS ON THE USE OF THE OGUR - ROSEN PROCEDURE—

It is apparent that the Ogur - Rosen procedure has seldom proved adequate for determining nucleic acids in biological materials. The most frequent cause for dissatisfaction has been incomplete extraction of RNA by the cold PCA treatment. The efficiency of RNA removal varies from tissue to tissue, and it has been suggested by Martin and Morton[279] that the PCA-resistant RNA may be of a different kind. Support for this view comes from examination of the rate of incorporation of radioactive phosphorus by the RNA of mouse embryo and of rat liver; the RNA fraction extractable from these tissues with cold PCA was found to have incorporated ^{32}P less extensively than the RNA that resisted extraction.[355] Further, study of the RNA of plant root tips[75] has shown that the portion extractable with cold PCA differs in base composition from the non-extractable RNA. This difference in base constituents has also been observed in RNA isolated from amphibian eggs and then immersed in 0·5 N PCA; part of the RNA immediately became acid-soluble, and this material differed in base components from the remainder.[103]

A less commonly reported source of difficulty with the Ogur - Rosen procedure has been partial extraction of DNA by the cold PCA treatment. The extent of this source of error does not appear to have been gross. However, attempts to improve the recovery of RNA during the first stage of the Ogur - Rosen procedure by raising the temperature of extraction with PCA have frequently been defeated because of partial solubilisation of the DNA at the higher temperature. A claim by Berenbom and Peters[366] to have obtained satisfactory separation of RNA from DNA by subjecting the tissue to three 20-minute extractions with N PCA at 25° C should be carefully evaluated, since experience obtained with PCA extraction at 4° C shows that tissues differ widely in the resistance of their contained RNA to PCA extraction.

Finally, it should be pointed out that the conditions used by Ogur and Rosen for extracting DNA (0·5 N PCA at 70° C for plant material and 1 N PCA at 80° C for animal tissues) are similar to those used in the Schneider hot PCA procedure for simultaneous extraction of both nucleic acids. Consequently, the criticism already made of the Schneider procedure as a method of extracting DNA will also apply to the use by Ogur and Rosen of hot PCA as an extractant of DNA.

4. OTHER PROCEDURES

From time to time, procedures other than the three already discussed have been devised for determining nucleic acids in biological material. Most of these have not been thoroughly evaluated, but it is conceivable that some might be of use when the recognised methods of nucleic acid analysis have proved inapplicable.

These less commonly used procedures will be considered under four headings: (*a*) extraction of nucleic acids with salt solutions; (*b*) other extraction procedures; (*c*) determination of nucleic acids by isolation of uracil and thymine; and (*d*) miscellaneous procedures connected with nucleic acid determinations.

EXTRACTION OF NUCLEIC ACIDS WITH SALT SOLUTIONS—

Several early investigators[375,376,377,378] in the field of nucleic acid studies observed that strong salt solutions could be used to extract the nucleic acids from tissues without undue degradation. The differing solubilities of RNA and DNA in salt solutions were made the basis of a non-quantitative separation procedure[379]; cold M sodium chloride was used to dissolve deoxyribonucleoprotein, and then the RNA was extracted with boiling 10 per cent. sodium chloride solution, which denatures the protein of the tissue and takes out both RNA and DNA, if present. This method has not been explored on a quantitative basis. The usual procedure for extracting nucleic acids with salt solutions is to treat the lipid-free tissue with 10 per cent. sodium chloride solution at 100° C for one hour or longer to obtain the two nucleic acids simultaneously. These can be precipitated from the extract by adding 2 volumes of ethanol[107] or lanthanum,[380,381] especially in the presence of ethanol[220] or cupric salts,[382] or by adjusting the extract to pH 1.[383,384] The precipitated nucleic acids can then be subjected to analysis for pentose and deoxypentose content,[220] separated by the Schmidt - Thannhauser alkaline-digestion procedure[25,32,384] or hydrolysed to provide the bases.[384]

Although hot sodium chloride extraction has most frequently been used to obtain nucleic acids relatively free from contamination, so that their uptake of radioactive precursors can be examined, it has been employed on several occasions with a view to quantitative measurement of nucleic acids in tissues. In an early series of studies on the distribution of RNA and DNA in tissues, Davidson and Waymouth[220] extracted the materials with hot 10 per cent. sodium chloride solution, precipitated the nucleic acids with lanthanum in the presence of ethanol and measured the amounts of RNA and DNA by sugar determinations. Davidson[385] did not regard this procedure as giving more than an approximate determination of tissue nucleic acid concentrations. However, Ada and Perry[46] considered hot 10 per cent. sodium chloride solution to be the best method for removing all the RNA from influenza virus, and Martin and Morton[279] also found that 10 per cent. sodium chloride solution, buffered at pH 4 to reduce extraction of tissue contaminants, could remove nucleic acids completely from beet petioles. In both of these studies, extraction of the sodium chloride residue with hot PCA failed to reveal significant amounts of nucleic acids absorbing at 260 mμ. Smillie and Krotkov[32] extracted samples of Euglena with 10 per cent. sodium chloride solution and recovered more than 90 per cent. of the total nucleic acid in the organism; however, a similar study on Chlorella resulted in almost complete failure to extract DNA with hot salt solution. One factor influencing extractability of DNA was brought to light by Lundin.[25] He considered that tissue samples treated with cold TCA and then with lipid solvents might still remain sufficiently acid to result in degradation of DNA when the tissue residue was extracted with hot sodium chloride solutions. He therefore buffered the first lipid solvent with sodium acetate, as suggested by Marko and Butler,[56] and his recoveries of DNA from liver then increased four-fold. Using this procedure he observed that less than 10 per cent. of the tissue nucleic acids remained unextracted by hot sodium chloride solution. Schmidt[386] has drawn attention to another possible factor preventing successful extraction of nucleic acids with salt solutions, namely, high concentrations of calcium ions, such as might occur in the mammary gland; this would render the nucleic acids less extractable, but does not seem to have impaired extraction of DNA from mammary gland or from the "microsomes" of milk.[360]

Hammarsten's[382] method of nucleic acid extraction and analysis may be regarded as a variant of the hot salt extraction method. After removing lipids from the tissue, he extracted the residue with a boiling solution of saturated sodium chloride - ammonium sulphate to which urea had been added. After several such extractions, the nucleic acids were precipitated from the combined extracts with copper sulphate. The isolated nucleic acids can then be separated by the phenol procedure[382] or by the Schmidt - Thannhauser method.[250,299] In the analysis of human placental tissue, Brody[299] found that the Hammarsten procedure gave values similar to those obtained by the Schneider but somewhat lower than those by the Schmidt - Thannhauser method; for complete recovery of RNA, Brody found that thirteen extractions with the hot salt - urea solution were necessary.

Manoïlov and Orlov[387] have used a method of obtaining nucleic acids from animal tissues in which extraction with saturated salt solution is preceded by treatment with 0·2 per cent. sodium hydroxide solution. Addition of acid to the extract caused partial precipitation of the nucleic acids; the precipitate is said to contain "stable" DNA, whereas the supernatant liquid after acidification contains RNA and "labile" DNA, which are then separated by alkaline digestion. This procedure has not been correlated with conventional methods of analysis.

OTHER PROCEDURES FOR EXTRACTING NUCLEIC ACIDS—

Several authors have combined two procedures in an attempt to obtain a satisfactory solution to some analytical problem. von Euler and Hahn[210,388] used cold 10 per cent. sodium chloride solution and then extraction with 0·2 N sodium hydroxide in the cold and achieved complete extraction of RNA from animal tissues. DNA was only partly removed from the tissue by this procedure; in order to obtain the DNA quantitatively, a separate portion of tissue was heated at 100° C with 0·5 N sodium hydroxide, and the DNA was then precipitated by acidification, thereby utilising the principle of Schmidt and Thannhauser to remove the RNA. In a series of studies on plant and mammary gland nucleic acids, Morton and his colleagues[279,360] eventually devised a procedure in which the tissue was first treated with cold PCA to remove part of the RNA, as in the Ogur - Rosen method, and was then extracted with 10 per cent. sodium chloride solution at 100° C to remove the remaining RNA and the DNA. Logan, Mannell and Rossiter[17] used both the Schmidt - Thannhauser and the

Schneider TCA procedures to obtain a composite picture of the nucleic acid fractions of nervous tissue.

Other procedures for nucleic acid extraction have been examined. Solomon[188,358] observed that phenol extracted more RNA but less DNA from the hen's egg than did hot PCA. Enzymic extraction of nucleic acids from plant tissues has been described,[389] but Ts'o and Sato[351] were unable to obtain satisfactory results by this procedure. However, Edström[373,390] has used ribonuclease to extract RNA from single nerve cells as a preliminary to determining their RNA content by a micro-spectrophotometric technique. Pigon and Edström[391] have extended the enzymic extraction technique to measurement of nucleic acid changes in a ciliate, for both RNA and DNA.

DETERMINATION OF NUCLEIC ACIDS BY ISOLATION OF URACIL AND THYMINE—

Since uracil is contained only in RNA and thymine in DNA, the quantitative isolation of these two bases should provide a measure of the RNA and DNA contained in a tissue; for absolute values, the base composition of the nucleic acids of the tissue under examination must be known. The use of this principle in conjunction with the RNA and DNA fractions of the Schmidt - Thannhauser procedure has already been discussed. It is possible, however, to isolate the bases directly from the tissue without preliminary separation of the nucleic acids. Jervell, Diniz and Mueller[392,393] digested the tissue residue, after washing in acid and lipid solvents, in 98 per cent. formic acid and then separated uridine and thymine by a combination of ion-exchange and paper chromatography; the yield of uridine had to be corrected for a 30 per cent. loss in the course of the procedure. Dutta[120] used a similar technique to recover the uracil and thymine from hot PCA extracts of tissues and obtained analyses of liver that differed by about 10 per cent. from those by the Schmidt - Thannhauser and Schneider procedures. McDonald[394] experienced difficulty in applying the conventional methods of analysis to rumen contents, but was able to obtain an estimate of total nucleic acid content by hydrolysing the lipid-extracted material with N hydrochloric acid at 100° C to release the purine bases, which were precipitated as their silver salts and purified by paper chromatography.

Isolation of bases has been combined with isotope dilution to obtain a measure of tissue RNA and DNA. Solomon[188,358] extracted the nucleic acids from hens' eggs with hot PCA or with phenol and measured the RNA and DNA in the extracts by dilution with ^{14}C-uracil and ^{14}C-thymine. Two groups,[395,396] studying the RNA content of enucleated specimens of Acetabularia, added ^{14}C-labelled purine bases to their material and hydrolysed the samples with N hydrochloric acid at 100° C to isolate the purine bases of RNA, on which measurements of isotope dilution were then made.

Finally, thymine has been measured directly on saline homogenates of mammalian tissues by using a specific fluorimetric procedure.[254] The results were mostly in close agreement with similar determinations made on extracts prepared by the Schneider hot TCA modification of the Schmidt - Thannhauser procedure[171]; in some tissues the values by direct thymine assay were considerably higher, but in view of the criticisms made earlier in this review of the use of hot acid to extract DNA, the TCA-extracted DNA value is not necessarily the final criterion of accuracy.

It is also appropriate to mention here a method for direct isolation of ribonucleotides from tissues without prior removal of acid-soluble small molecules; it has been used by Kemp and Allen[397] to determine the RNA in specimens of pancreas. The untreated tissue was digested in piperidine, which released mononucleotides from RNA by alkaline hydrolysis. After precipitation of DNA by acidification, the acid-soluble fraction was subjected to chromatography in order that the 2′- and 3′-mononucleotides arising from RNA hydrolysis might be separated from the 5′-nucleotides and the 2′,5′- and 3′,5′-diphosphonucleosides arising from the acid-soluble free nucleotides of the tissue. The RNA content of pancreas estimated from summing its 2′- and 3′-nucleotides gave a value 82 per cent. of its RNA content determined by a modified Schmidt - Thannhauser procedure.[73]

MISCELLANEOUS PROCEDURES CONNECTED WITH NUCLEIC ACID DETERMINATIONS—

To complete the survey, mention should be made of several procedures and observations not so far considered in this review, but of possible use to future investigators engaged in determining nucleic acids in biological materials.

In connection with the separation of RNA from DNA, Aldridge[398] has observed that the nucleic acids are precipitated by indium chloride and that either RNA or DNA can be precipitated selectively by varying the conditions of pH and ionic strength. It has also been noted[149,399] that RNA and DNA can be separated by electrophoresis on paper.

Methods of determining the nucleic acids based on properties of the whole molecule have been described. The nucleic acid content of pure solutions can be measured by polarography[400] and by the refractive index.[401] Fersini[402] has used the binding of methyl green by DNA as a method of determination in tissue extracts; the amount of dye not adsorbed is inversely proportional to the DNA content of the sample. A method of DNA determination based on its inhibitory action on the growth of the ciliate *Glaucoma pyriformis* has been described.[403]

Finally, methods proposed for the chemical determination of the purine and pyrimidine bases of RNA not mentioned here will be found in a comprehensive review by Schmidt.[404] Measurement of components of DNA and RNA by microbiological assay have been described and are reviewed in detail by Miller.[292] They provide a sensitive and specific method of measurement, but involve more elaborate organisation than the common chemical procedures.

GENERAL RECOMMENDATIONS FOR NUCLEIC ACID DETERMINATION

From this survey of analytical procedures, it is apparent that none has yet been devised that is infallible for nucleic acids. The three main methods—those of Schmidt - Thannhauser, Schneider and Ogur - Rosen—differ, however, in their liability to error. Most users of the Ogur - Rosen method have failed to obtain the complete separation of RNA from DNA claimed for it. The Schneider method suffers from the disadvantage that, if the conditions of hot acid extraction of the nucleic acids are not optimal, recovery of DNA may be incomplete either because of inadequate extraction or because of destruction of deoxyribose. It is thus unwise to apply the Schneider procedure to an untried tissue without providing some guarantee that no serious underestimate of its DNA content will result. On the other hand, hot acid extraction is a convenient and satisfactory method of obtaining RNA; in conjunction with the use of ultra-violet absorption to measure the extracted RNA, it provides a rapid means of measuring the amount of RNA in, say, cytoplasmic particles not containing DNA.

Of the three major procedures, the Schmidt - Thannhauser method in a modified form seems least subject to analytical error and therefore the most suitable to recommend as a first choice. Few users of this procedure have experienced difficulty in determining DNA, but the measurement of RNA in the acid-soluble fraction has proved less satisfactory. The use of phosphorus determinations on the acid-soluble fraction as a measure of its RNA content—the original procedure—is not satisfactory, and the determination of ribose by the orcinol procedure is sometimes liable to large errors due to the presence of other carbohydrates. Determination of RNA is best achieved by shortening to 1 hour the period of alkaline digestion of the tissue residue and then measuring the ultra-violet absorption of the acid-soluble fraction of the digest. Under these conditions, errors in ultra-violet absorption due to protein-degradation products are minimal, but this relative absence of protein should be confirmed by determining protein and by examining the ultra-violet spectrum of the acid-soluble fraction. A modified Schmidt - Thannhauser procedure embodying these and other recommendations is shown in Fig. 4.

The choice of method in relation to the amounts of material available is largely determined by the sensitivity of the final determination of RNA or DNA chosen. Methods of DNA determination by recently developed fluorimetric procedures offer a highly sensitive means of analysis on a few cells; for RNA, Edström[390] has provided a method applicable to single nerve cells. Whatever the method used, the choice of standards for the measurement of RNA and DNA is important. Ideally, this should be a sample of RNA or DNA isolated in pure form from the tissue on which the determinations are being performed. Thus, orcinol reacts mainly with the purine ribosides, so that variations in the proportion of purine bases in RNA will influence the result of this test if a suitable RNA standard is not employed. Similarly, isolation of thymine or uracil as a measure of tissue DNA or RNA demands a knowledge of the base ratios of the nucleic acids in the tissue.

Finally, tissues vary in the difficulty they present for determining nucleic acids. Plant tissues and some bacteria have proved rather intractable on account of the amount of

polysaccharide, metaphosphate and so on they contain and, with the Schmidt - Thannhauser procedure, because of lack of complete solubility in alkali. Investigators who are studying such material might profit by reading the papers of Smillie and Krotkov,[32] Sherratt and Thomas[123] and Morton and Martin[279] on their studies of plant tissue, Juni, Kamen, Reiner and Spiegelman[138] on yeast, Winder and Denneny[136] on the subject of bacteria, Solomon[358] on embryonic tissues and eggs and Logan, Mannell and Rossiter[17] on nervous tissue. These publications may help to suggest other approaches to meet analytical difficulties.

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Elimination of Interfering Citrate and Mineral Acids in the Spectrophotometric Determination of Phosphorus in Fertilisers*

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Existing methods for destroying citrate by wet-ashing procedures have been examined; more detailed methods were needed to give reproducible amounts of mineral acid in the residue after destruction of the citrate. Different ways of removing interference by acid with development of colour have also been investigated. The best way was found to be by partial neutralisation of the extra acid after addition of the molybdovanadate reagent solution. Application of the method to the determination of total P_2O_5 is described.

Commercially, phosphorus in fertilisers is determined by treating samples with water and neutral or alkaline solutions of ammonium citrate (or citric acid for samples of basic slag), and the phosphorus content of the extract is then determined; this is carried out in accordance with official methods, which differ in various countries. With the trend towards more rapid methods of analysis, the spectrophotometric molybdovanadate procedure for determining phosphorus has evoked great interest and has been adopted as official in the U.S.A. and the U.K. However, in this method, the presence of citrate ion above a certain limit interferes with development of the colour, and destruction of the citrate is the logical way to remove such interference and to make possible general adoption of the spectrophotometric procedure. Citrate can be destroyed by a wet-ashing procedure,[1,2,3,4] but when this technique was applied, a more detailed study was found necessary to attain the highest possible accuracy.

Spectrophotometric methods have often been declared unreliable, but the development of difference spectrophotometry has made possible the attainment of as high a precision as is achieved by volumetric analysis, provided that the technique used takes into account possible sources of error. A general treatment of the factors influencing precision in a form adapted to practical application has already been made.[5]

In this work, all optical-density measurements were made at 4200 A with a Beckman B spectrophotometer and 10-mm glass cells, and the solutions used were prepared as described below.

Reagent solution—Dissolve 20 g of ammonium molybdate in 400 ml of water at 50° C and 1 g of ammonium vanadate in 300 ml of water; acidify the latter solution with 140 ml of nitric acid, sp.gr. 1·40. Slowly and with constant stirring, add the molybdate solution to the acid vanadate solution, and dilute the mixture to 1 litre.

Standard phosphate solution—Dry potassium dihydrogen orthophosphate for 2 hours at 105° C, grind it, and dry for a further 2 hours at 105° C. Dissolve 0·4793 g of the dried salt in water, add a few drops of chloroform as preservative, and dilute in a calibrated flask to 1 litre.

$$1 \text{ ml} \equiv 0{\cdot}25 \text{ mg of } P_2O_5.$$

Reference solution—By means of a piston burette, measure 20·00 ml of standard phosphate solution into a 100-ml calibrated flask, add 30·0 ml of the reagent solution, and dilute to the mark. This solution[5,6] contains 5 mg of P_2O_5.

Note—The reference solution should be prepared at the same time as colour is developed in the sample solution, and both should be set aside for at least 10 minutes to attain complete development of the colour.

Interference from citrate

The effect of citrate ion on development of colour is shown in Fig. 1, in which the citrate content of the final solution is plotted against the value of d (the difference between the optical

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densities of the sample and reference solutions). It can be seen that, in a final coloured solution containing 5 mg of P_2O_5 per 100 ml, there is no interference when the citrate content is 150 mg or less. At contents above 150 mg, interference is apparent and is more pronounced when the citrate is added as citric acid than when it is added as Petermann's solution[7] (a solution of ammonium citrate made alkaline with ammonia).

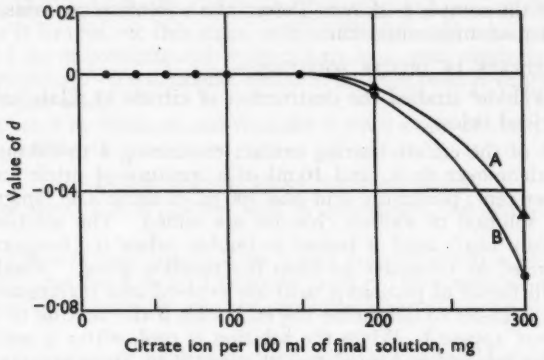

Fig. 1. Interference by citrate ion with colour developed by 5 mg of P_2O_5 in 100 ml of final solution (d = difference between optical densities of sample and reference solutions): curve A, citrate added as Petermann's solution; curve B, citrate added as citric acid

For an aliquot of sample containing 5 mg of P_2O_5, when the water- and citrate-soluble P_2O_5 are extracted in accordance with official Swedish procedure,[7] interference will occur with all fertilisers containing less than 21 per cent. of P_2O_5, and the limit will be 27 per cent. of

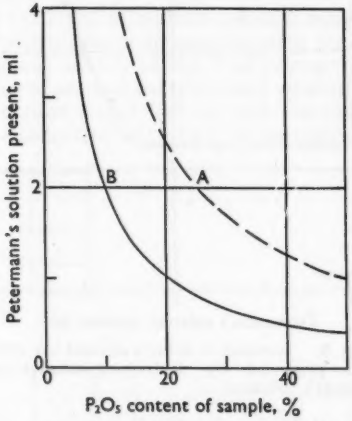

Fig. 2. Relationship between P_2O_5 content of sample and amount of Petermann's solution in aliquot containing 5 mg of P_2O_5 when official Swedish method[7] is used: curve A, citrate-soluble extract; curve B, water- and citrate-soluble extract

P_2O_5 when an aliquot containing 6·5 mg of P_2O_5 is taken. In determinations of citrate-soluble P_2O_5, interference will occur with even the high-grade fertilisers. However, there will be no interference with the determination of P_2O_5 soluble in citric acid. This means

that, in practice, citrate ion will generally interfere. Procedures for overcoming or compensating for interference from citrate are limited in their application, except for those involving destruction of the citrate, and only these latter were studied.

In an aliquot containing 5 mg of P_2O_5, the accompanying amount of Petermann's solution will depend on the percentage of P_2O_5 in the fertiliser and on the method of extraction. In Fig. 2 the relationship between the accompanying amount of Petermann's solution and the P_2O_5 content of the sample is shown; Petermann's solution contains, per ml, 156 mg of citrate ion and 54 mg of ammonium ion.

DESTRUCTION OF CITRATE IN DILUTE SOLUTION—

Brabson and Wilhide[3] studied the destruction of citrate in dilute solution and devised the procedure described below.

> An aliquot of the citrate-bearing extract containing 4 to 6·5 mg of P_2O_5 is placed in a 125-ml Erlenmeyer flask, and 10 ml of a mixture of nitric and perchloric acids (300 ml of 70 per cent. perchloric acid *plus* 700 ml of nitric acid, sp.gr. 1·40) and 5 ml of a 20 per cent. solution of sodium chlorate are added. The solution is warmed on a hot-plate (medium heat) until it begins to bubble, when the temperature is decreased. The flask is moved as necessary to keep the reaction going. Finally, the solution is evaporated until fumes of perchloric acid are evolved and maintained at this stage for 2 minutes. (It is easier to determine the end-point if the heating to fumes is continued until crystals just appear.) When the solution is cool, water is added to dissolve the crystals, and the solution is heated for 10 minutes to hydrolyse non-orthophosphates. It is then transferred to a 100-ml calibrated flask and diluted to about 50 ml, 30 ml of molybdovanadate reagent solution are added, and the mixture is diluted to the mark.

When this method is used, citrate is destroyed, but the introduction of acid affects the acidity of the final solution, thereby interfering with the intensity of colour. The problem has therefore changed in character.

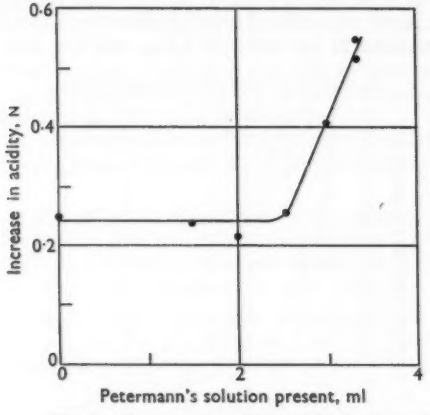

Fig. 3. Increase in acidity of final coloured solution produced by destroying citrate in Petermann's solution

Brabson and Wilhide's method was applied to a series of solutions each containing 20 ml of water and a different amount of Petermann's solution, and the residual amounts of acid were determined. The results are shown in Fig. 3, from which it can be seen that this amount is constant in presence of less than 2·5 ml of Petermann's solution, but is considerably increased when more Petermann's solution is present. As more than 2·5 ml of Petermann's solution is frequently used, the ammonia must be destroyed in order to obtain a reproducible amount of acid. This complication was not reported by Brabson and Wilhide,[3] who, however, used a neutral solution of ammonium citrate and so introduced less ammonia. With 2·5 ml of Petermann's solution, 135 mg of ammonium ion are introduced, of which 91 mg remain after destruction of citrate; with 3·3 ml of Petermann's solution, the corresponding figures

are 178 and 157 mg. Some destruction of ammonium ion therefore occurs when the citrate is destroyed.

It was thought that, in dilute solution, ammonium ion might be destroyed by oxidation to nitrous oxide in a mixture of hydrochloric acid, a little nitric acid and perchloric acid,[8] and, after different procedures for destroying ammonium ion had been tested, the method described below was adopted.

Proceed with the destruction of citrate described above until the solution is colourless, and allow it to cool; at this stage, sufficient nitric acid remains. Dilute with 5 ml of water, add 1 ml of concentrated hydrochloric acid, and continue in accordance with the original procedure. No ammonia remains, and the residue contains 25 milli-equivalents of perchloric acid.

The results reported by Brabson and Wilhide[3] showed a bias of about 0·5 per cent. caused by the acid in the residue.

Interference from mineral acids

If, during treatment of the sample, acid is introduced, the remedies listed below are conceivable.

1. With the reference solution the usual reagent solution is used, but with the sample solution another reagent solution is used. The combined acid contents of this reagent solution and the sample solution should be about the same as that of the reference solution.

2. The same reagent solution is used with both sample and reference solutions, but to the reference solution is added as much acid as is introduced with the sample. This can be done in two ways:

(*a*) the acid in the reagent solution, together with that in the sample, must give the usual acidity in the final coloured solution or

(*b*) the ordinary reagent solution is used.

3. The acid in the sample is neutralised; this could be done either (*a*) before or (*b*) after addition of the reagent solution.

Investigation of possible remedies—

In this work, 20 ml of standard phosphate solution were present in both sample and reference solutions. When the ordinary reagent solution was used and no extra acid was introduced with the sample, the acidity of the final coloured solution was 0·61 N.

To study remedy 1, different amounts of nitric acid were added to the standard phosphate solution, and the reagent solution added had an acid content that made the final solution 0·35 N. These colours were compared with that of an ordinary reference solution. From

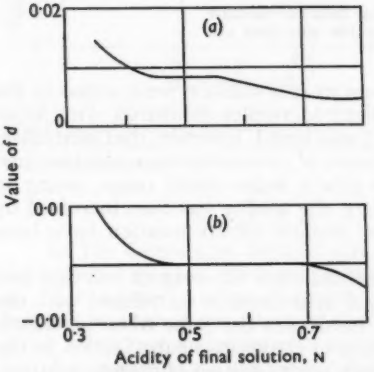

Fig. 4. Effect of acidity of final coloured solution on difference, *d*, between optical densities of sample and reference solutions: (*a*), remedy 1 used; (*b*), remedy 2 (*a*) used

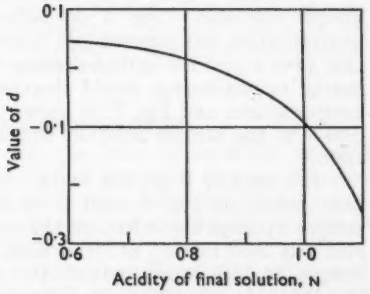

Fig. 5. Effect of acidity of final coloured solution on difference, *d*, between optical densities of sample and reference solutions when remedy 2 (*b*) is used

Fig. 4 (*a*) it can be seen that this remedy cannot be applied; a value of *d* of 0·001 means a relative analytical error of 0·1 per cent.

For remedy 2 (*a*) the sample solutions were prepared as described above and the reference solution by adding 26 milli-equivalents of nitric acid to the standard phosphate solution. A special reagent solution was used to make the final coloured solution 0·61 N in acid. Fig. 4 (*b*) shows that this remedy is useful if the sample introduces 10 to 35 milli-equivalents of acid into the final solution. This remedy was suggested by Brabson and Wilhide[3] and later adopted officially.[9] A reagent solution having such low acidity, however, is stable only for about 1 week.

For remedy 2 (*b*), the usual reagent solution was used, and, in preparing the reference solution, 25 milli-equivalents of nitric acid were added to the standard phosphate solution before the reagent solution was added. The sample solution was prepared in the same way, except that the amount of nitric acid was varied. Fig. 5 shows that this remedy cannot be applied.

Deviations from the usual conditions were next studied, and three of the sample solutions were treated in three ways: (*i*) nitric acid was added before the reagent solution (branch A in Fig. 6); (*ii*) sodium hydroxide solution was added before the reagent solution (branch B) and (*iii*) sodium hydroxide solution was added after the reagent solution (branch C). It was found that the sample solution must not be alkaline before addition of the reagent solution.

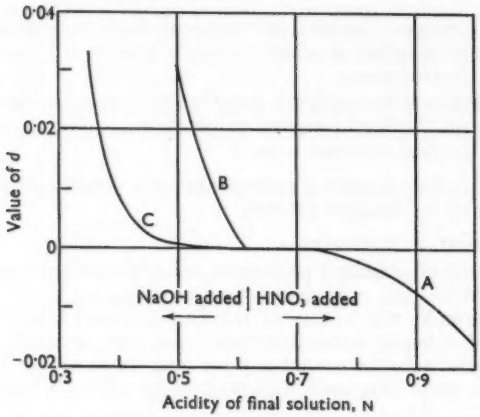

Fig. 6. Effects of deviation from usual conditions when nitric acid and sodium hydroxide solutions are added

To study remedy 3 (*a*), different amounts of sodium nitrate solution were added to the sample solutions. Fig. 7 (*a*) indicates no improvement in results compared with when neutralisation was omitted (see branch A in Fig. 6). It was found, however, that neutralisation gave a positive optical-density difference and omission of neutralisation a negative one; partial neutralisation would therefore be expected to give a wider useful range, owing to compensation, and Fig. 7 (*b*) shows that this is so. Here, the acidity has been increased by 0·05 N in the sample solution and different amounts of sodium nitrate solution have been added.

For remedy 3 (*b*), the extra nitric acid was neutralised after the reagent solution had been added (see Fig. 8), and up to 30 milli-equivalents of acid could be introduced with the sample without any effect on the optical density; the result was the same when perchloric acid was used instead of nitric acid. To study the effect of erroneous neutralisation in the sample, 24 milli-equivalents of nitric acid were added, and, after addition of reagent solution, this acid was neutralised by different amounts of sodium hydroxide solution. Fig. 9 shows that an excess of sodium hydroxide is harmful, but that a deficiency of up to 10 milli-equivalents is not. During neutralisation, the temperature of the solution increases by several degrees, so that it is necessary to cool before diluting to the mark.

Of the possible remedies, 3 (*b*) is the best choice. An excess of sodium hydroxide causes less harm than with remedy 3 (*a*), and, compared with remedy 2 (*a*), the ordinary and stable reagent solution is used. It is advisable to add about 5 milli-equivalents of sodium hydroxide less than the amount of acid introduced with the sample. This means that, in the modified version of Brabson and Wilhide's method for destroying citrate, 9 ml of 2 N sodium hydroxide must be added to the sample solution before dilution to the mark.

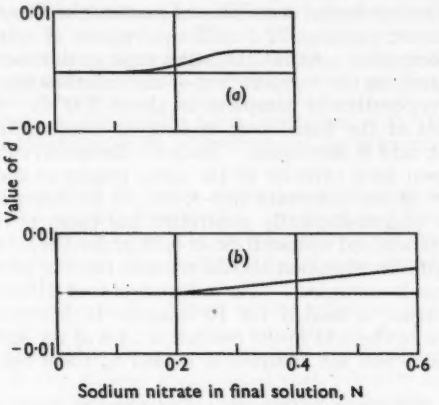

Fig. 7. Effect on difference, *d*, between optical densities of sample and reference solutions of (*a*) adding sodium nitrate and (*b*) adding sodium nitrate when the acidity of the sample solution had been increased by 0·05 N

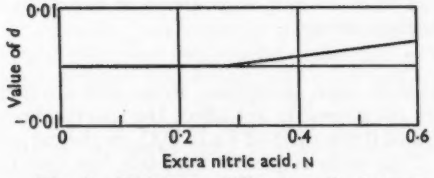

Fig. 8. Effect on difference, *d*, between optical densities of sample and reference solutions of extra nitric acid when neutralised after addition of reagent solution

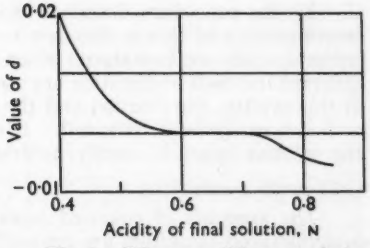

Fig. 9. Effect on difference, *d*, between optical densities of sample and reference solutions of acidity of final coloured solution when an extra 24 milli-equivalents of acid are introduced and neutralised by different amounts of sodium hydroxide

Destruction of citrate in concentrated solution

In the destruction method already described, the sample must be carefully watched during fuming, as it contains perchlorates and there is risk of explosion if it is left alone on the hot-plate. It was therefore desirable to find an alternative method having no such hazards; a method described by Gehrke and Johnson,[4] who used a concentrated mixture of nitric, perchloric and sulphuric acids, seemed to be adequate.

The acid mixture was prepared by mixing 10 parts by volume of 70 per cent. nitric acid, 4 parts of 70 per cent. perchloric acid and 2 parts of 96 per cent. sulphuric acid, the sulphuric acid being added to the nitric acid and mixed well before addition of the perchloric acid. To an aliquot of the citrate-bearing extract containing 4 to 6·5 mg of P_2O_5 in a 100-ml Erlenmeyer flask were added 5 ml of the acid mixture, and the solution was heated on a hot-plate.

First, water vapour was evolved, and, when the temperature of the solution had reached about 140° C, destruction of citrate began; after continued heating, the discoloration caused by charring completely disappeared.

After destruction of the citrate, some acid remains in the residue. For reasons already made clear, this amount of acid must be reproducible in order to obtain correct conditions for development of colour. However, Gehrke and Johnson's method did not give reproducible amounts of residual acid. Further experiments were carried out with samples containing 20 ml of standard phosphate solution and different amounts of Petermann's solution. A 5-ml portion of the acid mixture contains 22·5 milli-equivalents of sulphuric acid, 47·5 of nitric acid and 14·5 of perchloric acid. About the same time as destruction of citrate begins, the nitric acid evaporates, and, as the temperature of the solution increases, the perchloric acid also evaporates; this evaporation is complete at about 200° C. The perchloric acid tends to condense on the walls of the flask, and, as long as condensation takes place, complete evaporation of perchloric acid is impossible. Such condensation can be avoided by surrounding the flask with an open steel cylinder of the same height as the flask; further control of the surface temperature of the hot-plate was found to be essential, the best results being obtained at 240° C. A thermostatically controlled hot-plate is recommended. The total time for destruction of citrate and evaporation of acid under these conditions was 65 minutes. If, after this period, slight discoloration should remain, heating must be continued for a few minutes until the solution is colourless. The solution is then allowed to cool, 20 ml of water are added, and the mixture is heated for 10 minutes to hydrolyse non-orthophosphates. For partial neutralisation of the acid in the residue, 3·7 ml of 2 N sodium hydroxide are added after the reagent solution, and the solution is cooled to room temperature before dilution to the mark.

With this modified technique, citrate was destroyed in more than forty samples, each containing a different amount of Petermann's solution up to 4 ml and 5 mg of P_2O_5. The mean value of d, the difference between the optical densities of sample and reference solutions, was 0·000, with a mean deviation of 0·001; the method therefore involves no bias.

Application to determination of total P_2O_5—

In the procedure described above, citrate interference is converted into mineral acid interference, and this is eliminated. In the determination of total P_2O_5, further amounts of mineral acids are introduced when the aliquot is taken. For different kinds of material, different methods of digestion are used, and each method gives a certain acidity in the extract. If this acidity, the dilution and the size of the aliquot are known, the additional amount of acid can easily be determined. If there are more than 10 milli-equivalents in the aliquot, the solution must be partly neutralised as described above.

Coloured impurities—

The amounts of coloured metal ions in extracts from phosphate rocks and products manufactured therefrom are generally so low that these ions do not affect the spectrophotometric measurement. The most common is iron, and if the ratio of Fe to P_2O_5 in the extract is less than 0·5, interference can be neglected. If the ratio is greater than 0·5, the spectrophotometric method cannot be recommended for determining the P_2O_5, nor can it be recommended if the extract contains impurities forming coloured complexes with the molybdovanadate reagent.

I thank Mr. Kornél Horváth, who, with great care and interest, carried out all the measurements.

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The Estimation of Cellulose and Household Coal in Town-refuse Compost

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After treatment with Scharrer and Kürschner's reagent, the cellulose in compost is determined by direct titration with potassium dichromate solution; the presence of household coal does not interfere. By determining the loss on ignition of the residue after treatment with the reagent, the household-coal content can easily be ascertained by difference.

In Gerretsen and Campen's method for determining household coal in town-refuse compost,[1] the sample is boiled with the reagent described by Scharrer and Kürschner.[2] This dissolves some of the organic substances, such as proteins and lignin, but not the cellulose and household coal. Some of the hemicelluloses are also dissolved, but it is not certain that the disintegration of hemicelluloses is quantitative.

Gerretsen and Campen suggested solution of the cellulose in Schweitzer's reagent, or hydrolysis of it by sulphuric acid, and then determination of the household coal by ignition of the residue in a muffle furnace; the decrease in weight was considered to represent the coal. In a parallel determination, the sample was treated with Scharrer and Kürschner's reagent, but not with Schweitzer's solution, and was then ignited; the loss on ignition represented the sum of the cellulose (perhaps together with some of the hemicelluloses) and household coal present. The difference between the results of the two determinations was claimed to represent the cellulose. The determination of cellulose content was consequently indirect and subject to all the uncertainties of such methods.

After further research at the Institute for Soil Fertility, a direct procedure for determining cellulose in town-refuse compost has been devised; in this method, the cellulose in the residue left after treatment of the sample with Scharrer and Kürschner's reagent is determined by modification of the dichromate-oxidation method described by Kurmies[3] and often used for determining organic matter in soil.

Experimental

Initially, pure cellulose (filter flakes, obtained from Messrs. Schleicher and Schüll) and ground coke were subjected to oxidation with dichromate by Kurmies' method; later, town-refuse compost and humic acid (obtained from E. Merck A.G.) were included in the experiments. Three factors were studied: (*a*) the effect of the concentration of sulphuric acid used, (*b*) the effect of treatment with Scharrer and Kürschner's reagent on the amount of cellulose recovered and (*c*) the percentage recovery of cellulose after oxidation by the dichromate solution.

Effect of concentration of sulphuric acid on oxidation of cellulose and coke by dichromate—

In each of a series of 250-ml calibrated flasks containing a known weight of sample were placed 25 ml of 2 N potassium dichromate and 40 ml of sulphuric acid of known concentration. Each flask was heated for 90 minutes in a bath of boiling water, with intermittent shaking, and, when cool, its contents were diluted to the mark and mixed. A 25-ml portion of this solution was transferred, by pipette, to a 250-ml beaker containing 25 ml of 0·22 N ferrous sulphate, a few drops of *N*-phenylanthranilic acid indicator solution were added, and the excess of ferrous sulphate was titrated with 0·1 N potassium permanganate until the colour changed from green to violet. A blank experiment was carried out concurrently. The results of these experiments are listed in Table I.

It is obvious from these results that, when the concentration of sulphuric acid is not greater than 15 per cent. w/w, oxidation of coke (and certainly of household coal) is negligible and will not interfere with the determination of cellulose in compost, whereas the cellulose was oxidised almost quantitatively.

TABLE I

EFFECT OF CONCENTRATION OF SULPHURIC ACID ON OXIDATION OF VARIOUS MATERIALS

Sample	Carbon content found by elemental analysis, %	Carbon content found in solution after oxidation with dichromate and— 96% w/w sulphuric acid, %	77% w/w sulphuric acid, %	58·5% w/w sulphuric acid, %	34·5% w/w sulphuric acid, %	25% w/w sulphuric acid, %	20% w/w sulphuric acid, %	15% w/w sulphuric acid, %
Ground coke ..	80·4	34·0	14·0	4·2	1·5	1·8	1·3	1·4
Cellulose ..	44·4*	42·4	41·4	41·6	40·7	40·8	40·8	40·8
Compost ..	24·2	20·8	18·5	15·8	13·7	13·3	13·1	12·8
Humic acid ..	48·8	48·7	40·8	34·1	28·8	—	—	26·0

* Theoretical value.

RECOVERY OF CELLULOSE AFTER TREATMENT WITH SCHARRER AND KÜRSCHNER'S REAGENT—

Different amounts of pure cellulose were boiled with Scharrer and Kürschner's reagent (see "Reagents," p. 823); blank tests, in which the cellulose was boiled with distilled water, were carried out concurrently. The residues were washed with water alone or with water and then acetone. The results, each of which is the mean of three determinations, are shown in Table II.

TABLE II

RECOVERY OF PURE CELLULOSE AFTER BOILING WITH SCHARRER AND KÜRSCHNER'S REAGENT

Cellulose taken, mg	Cellulose recovered after boiling with water; residue washed with water and acetone (A), mg	Cellulose recovered after boiling with Scharrer and Kürschner's reagent— residue washed with water and acetone (B), mg	residue washed with water only, mg	Ratio of A to B
50	50·3	45·9	—	1·089
100	100·7	93·1	91·3	1·074
200	196·4	181·6	181·0	1·082
			Mean	1·082

From the results, it is evident that cellulose is slightly attacked by the reagent, since recovery was almost complete in the blank tests. However, the amount of cellulose originally present can be derived by multiplying the amount recovered by 1·08; the value of this factor proved to be almost independent of the amount of cellulose taken.

OXIDATION OF CELLULOSE BY TREATMENT WITH DICHROMATE—

Pure cellulose was first boiled with Scharrer and Kürschner's reagent and then oxidised by potassium dichromate solution and acid; both concentrated and 15 per cent. w/w sulphuric acid were used. All experiments were carried out in triplicate, and the mean results are shown in Table III.

TABLE III

CELLULOSE OXIDISED BY DICHROMATE

Pre-treatment	Carbon oxidised* after treatment with potassium dichromate solution and— concentrated sulphuric acid, %	15% w/w sulphuric acid (A), %	Conversion factor (ratio of 44·44 to A)
None	42·35	40·80	—
Boiled with water	42·86	40·82	—
Boiled with Scharrer and Kürschner's reagent; residue washed with water	42·13	39·59	1·123
	41·34	39·64	1·121
Boiled with Scharrer and Kürschner's reagent; residue washed with water and acetone ..	41·72	39·72	1·119
		Mean ..	1·121

* Calculated from the weight of residue after the pre-treatment.

It can be seen from these results that, again, not all of the cellulose is oxidised, but the correct figure can be calculated by multiplying the amount found by 1·12. The results of these experiments were reproducible, and it was therefore considered justifiable to recommend the method described below for determining cellulose and household coal in town-refuse compost.

Method

Reagents—

Scharrer and Kürschner's reagent[2]—Prepare a solution of 70 per cent. acetic acid containing, per litre, 27·5 g of trichloroacetic acid and 68 ml of nitric acid, sp.gr. 1·4.

Acetone.

Sulphuric acid, 15 per cent. w/w—Add 94 ml of sulphuric acid, sp.gr. 1·84, to water, allow to cool, and dilute with water to 1 litre.

Potassium dichromate, 2 N—Dissolve 98 g of potassium dichromate in water, add 100 ml of sulphuric acid, sp.gr. 1·84, and dilute with water to 1 litre.

Ferrous sulphate, 0·22 N—Mix 60 g of ferrous sulphate heptahydrate with 120 ml of sulphuric acid, sp.gr. 1·84, and dilute with water to 1 litre.

Potassium permanganate, 0·1 N.

Indicator solution—Dissolve 200 mg of *N*-phenylanthranilic acid in 100 ml of a 0·2 per cent. w/v solution of sodium carbonate.

Pre-treatment of sample—

Weigh 500 mg of the ground and carefully mixed sample into a 300-ml Erlenmeyer flask. Add 80 ml of Scharrer and Kürschner's reagent, and boil under reflux for 90 minutes. Transfer the residue to a Gooch crucible (outside diameter 42 mm) containing a layer of about 5 g of asbestos that has been boiled with hydrochloric acid, washed with water until free from chloride and then ignited for several hours at about 900° C in a muffle furnace. Successively wash the residue, by percolation, with some of Scharrer and Kürschner's reagent, with water until free from acid and then with two 25-ml portions of acetone. Use this sample for the determination of cellulose *plus* household coal.

Similarly treat another sample of the ground compost, but use a sintered-glass crucible (porosity No. 4) instead of the Gooch crucible.

Procedure for determining cellulose *plus* coal—

Dry the Gooch crucible containing the washed residue overnight at 105° C, weigh, and heat in a muffle furnace at about 900° C for some hours. Allow to cool in an desiccator, and re-weigh; the loss on ignition, l, represents the cellulose *plus* coal in the sample. Calculate the content of cellulose *plus* household coal (A) in the compost from the expression—

$$A \text{ (uncorrected), } \% = \frac{100\,l}{0{\cdot}5}.$$

Procedure for determining cellulose—

Place the sintered-glass crucible containing the residue from pre-treatment of the second portion of sample in a wide-necked Erlenmeyer flask, and add 40 ml of 15 per cent. w/w sulphuric acid and 25 ml of 2 N potassium dichromate. Cover the neck of the flask with a watch-glass, and heat for 90 minutes in a bath of boiling water, with intermittent shaking. Concurrently, carry out a blank experiment. When cool, remove the crucible from the flask, rinse it carefully and add the rinsings to the contents of the flask. Wash the sintered-glass plate of the crucible, by suction, and again add the washings to the contents of the flask. Transfer the solution to a 250-ml calibrated flask, dilute to the mark, and carefully mix. By pipette, add 25 ml of this solution to 25 ml of 0·22 N ferrous sulphate in a 250-ml beaker, add a few drops of the indicator solution, and titrate the excess of ferrous sulphate with 0·1 N potassium permanganate until the colour changes from green to violet. Let the titre found *minus* the blank titre be b, and calculate the uncorrected content of cellulose in the sample (B) from the expression—

$$B, \% = \frac{b \times N \times 10 \times 0{\cdot}003 \times 1{\cdot}12 \times 2{\cdot}25 \times 100}{0{\cdot}5}$$
$$= 15{\cdot}12\,Nb.$$

The content of household coal in the sample is the difference between A and B, and the corrected percentage of cellulose is $1{\cdot}08\,B$.

Application of the method

The proposed method was applied to samples of seven different batches of town-refuse compost; the results are summarised in Table IV. No analysis of variance was carried out

Table IV

Cellulose and household-coal contents of different composts

All results are expressed on the dry basis for the original sample

Sample No.	Sample designation	Carbon found by dichromate method multiplied by 1·12 (A), %	Uncorrected cellulose content* (B) %	Loss on ignition after treatment with Scharrer and Kürschner's reagent (C), %	Household-coal content,† %	Corrected cellulose content (B × 1·08), %
1	Schiedam No. 5; fresh ..	5·21, 4·99 (mean 5·10)	11·48	19·23, 19·20 (mean 19·21)	7·7	12·4
2	Venlo No. 1; fresh ..	3·48, 3·58 (mean 3·53)	7·94	17·57, 17·73, 17·03 (mean 17·44)	9·5	8·6
3	Venlo No. 7; fermented	1·68, 1·79 (mean 1·74)	3·92	18·11, 17·91 (mean 18·01)	14·1	4·2
4	Venlo No. 8; fresh ..	3·60, 4·05, 3·46 (mean 3·70)	8·33	26·28, 26·11 (mean 26·20)	17·9	9·0
5	Vam; fermented ..	2·23, 2·23 (mean 2·23)	5·02	30·12, 31·48 (mean 30·80)	25·8	5·4
6	Delft; fresh	10·75, 13·09, 11·76 (mean 11·87)	26·70	35·87, 35·87 (mean 35·87)	9·2	28·8
7	Delft; fermented ..	5·08, 4·71, 5·07 (mean 4·95)	11·14	32·44, 29·25, 30·37 (mean 30·69)	19·6	12·0

* Mean value in column A multiplied by 2·25.
† Mean value in column C *minus* value in column B.

on these results, as the number of replicate determinations was too small, but it can be seen that the spread was significant, since town-refuse composts are not particularly homogeneous.

From the results in Table IV, some other figures indicating the qualities of the composts have been derived, and these are shown in Table V.

Table V

Properties of analysed composts (derived from results in Table IV)

All results are expressed on the dry basis

Sample No. (see Table IV)	Corrected cellulose content (A), %	Household-coal content (B), %	Loss on ignition of untreated sample (C),* %	Useful organic-matter† content (D), %	Organic matter other than cellulose (D − A), %	Cellulose content of useful organic matter (100 A/D), %
1	12·4	7·7	39·9	32·2	19·8	38·5
2	8·6	9·5	30·5	21·0	12·4	41·0
3	4·2	14·1	27·3	13·2	9·0	31·8
4	9·0	17·9	39·5	21·6	12·6	41·7
5	5·4	25·8	37·2	11·4	6·0	47·4
6	28·8	9·2	58·1	48·9	20·1	58·9
7	12·0	19·6	35·7	16·1	4·1	74·5

* Corrected for carbon dioxide from carbonates.
† Organic matter considered useful as manure; it is gradually disintegrated by microbes and gives rise to humus. The figure is derived by subtracting the value in column B from that in column C.

The figures in column C of Table V represent the total combustible matter present in the samples, and the remarkably high variability of the household-coal content is mainly due to the difference between winter and summer batches of compost. The high content of cellulose in the organic matter, even for old fermented samples, is surprising, but the decrease

in cellulose content during fermentation of the fresh refuse is sufficiently evident to as be indicative of the extent of disintegration during fermentation.

Although the proposed method is reliable and can apparently provide valuable information, the results should still be compared with those found by Gerretsen and Campen's method.[1]

I thank Dr. F. C. Gerretsen for suggesting the study of this problem and for his valuable advice and criticism.

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A Routine Method for determining Caffeine in Coffee and Coffee Mixtures

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A spectrophotometric method is described for determining caffeine in coffee; the base-line procedure is used. Interfering impurities are removed from the samples by preferential extraction of caffeine with chloroform from an alkaline solution and subsequent passage of the extract through a column of activated alumina. Results obtained by this method compare favourably with those found by the official Bailey - Andrew procedure. The caffeine contents of coffees containing chicory are found by determining the nitrogen contents of the chloroform extracts. Simplicity and accuracy are the outstanding characteristics of the method.

Coffees are usually blended or adulterated with maize or chicory, and the aqueous extract and caffeine content of a sample usually give good indication of the degree of adulteration. In a control laboratory, many samples of coffee mixtures are analysed daily for caffeine, and for many years the Power - Chestnut[1] and Bailey - Andrew[2] methods recommended by the Association of Official Agricultural Chemists were used in this laboratory; these methods are satisfactory, but excessively time-consuming. Ishler, Finucane and Borker[3] described a spectrophotometric method for determining caffeine in coffee. They removed interfering impurities, such as trigonelline and chlorogenic acid, by treatment with heavy magnesium oxide and zinc ferrocyanide and sometimes also by oxidation with permanganate before the aqueous extracts were analysed spectrophotometrically. This procedure gave inconsistent results, particularly when applied to coffee mixtures, when oxidation by permanganate must be used. Because of these limitations, a routine method for determining caffeine in coffee was sought.

Development of method

It was hoped that complete extraction of caffeine from coffee could be effected by heating the sample under reflux with chloroform in the presence of alkali. This extraction procedure has the advantage that interfering substances, such as chlorogenic acid and trigonelline, are removed, as the former is "fixed" by the alkali and the latter is insoluble in chloroform. Other chloroform-soluble interfering substances were expected to be removed by passing the chloroform extract through a column of activated alumina. Studies of the ultra-violet absorption spectra of the diluted chloroform extracts showed that, except for those of samples

containing chicory, all the spectra had maxima at 277 mμ and were similar to that of caffeine itself. However, when the caffeine contents of these samples were calculated directly from the absorption at 277 mμ, the results were considerably higher than those obtained for the same samples by the official Bailey - Andrew procedure (see Table I).

TABLE I

CAFFEINE CONTENTS FOUND BY VARIOUS METHODS

Sample No.	Number of determinations by each method	Caffeine content found by— spectrophotometry without background correction— in chloroform, %	in water, %	calculation from nitrogen content, %	Bailey - Andrew method, %
Roasted coffee—					
1	3	1·94	2·01	1·70	1·60
2	3	1·87	1·84	1·52	1·45
Coffee mixed with maize—					
1	3	1·15	1·19	1·07	1·00
2	3	0·94	1·01	0·93	0·88
Coffee mixed with chicory—					
1	4	2·35	1·30	1·12	1·08
2	4	2·46	1·34	1·15	1·12

It was suspected that the interfering substances might be chloroform-soluble, and attempts were made to remove them by evaporating the chloroform extracts and dissolving the residues in water before spectrophotometry. Again, all the spectra except those from the samples containing chicory had maxima at 273 mμ and were similar to that of pure caffeine. The caffeine contents calculated directly from the absorption at 273 mμ agreed closely with those obtained from the chloroform extracts, except for the samples containing chicory (see Table I). These results indicated the existence of some irrelevant absorption, and this was confirmed by determining the nitrogen contents of the chloroform extracts, the values of which are also shown in Table I. Attempts were made to destroy these interfering substances by oxidation with permanganate in neutral medium as described previously,[3] but without success.

The fact that the maxima and minima of the spectra agreed closely with those of pure caffeine suggested that the spectra of the interfering substances were linear, or nearly so, and a study of extracts (in chloroform and water) obtained by the proposed procedure from roasted maize confirmed this suggestion. Effects of background absorption can be minimised by using the base-line procedure successfully employed by Morton and Stubbs[4] and McGillivray[5] for determining vitamin A in fish oils. Willits, Swain, Connelly and Brice[6] applied the same procedure to the determination of nicotine in tobacco.

Investigation has shown that this procedure can be applied successfully to both chloroform and aqueous extracts of coffee, and the results are in good agreement with those found by a modified version of the Bailey - Andrew procedure[7] (see Table II). This has led to the adoption of the method described below.

METHOD

PROCEDURE—

Weigh 1·0 g of roasted coffee or 0·5 g of soluble coffee, transfer to a 150-ml flask containing 3 ml of a 10 per cent. solution of ammonium hydroxide, add 80 ml of chloroform, and heat under reflux for 30 minutes. Pour the contents of the flask into a funnel plugged with cotton-wool, and allow the filtrate to run directly on to a column consisting of 10 g of alumina (previously heated to 800° C for 6 hours) packed in a 100-ml burette. Rinse the flask with more chloroform, pour the rinsings on to the column, and collect the eluate in a 200-ml calibrated flask.

Collect about 120 to 150 ml of eluate, which normally contains all the caffeine in the sample, and dilute to the mark with chloroform. By pipette, place a 5- or 10-ml portion of eluate in a 50-ml calibrated flask, dilute to the mark with chloroform, and calculate the caffeine content of the sample from the optical-density readings for this solution at 257, 277 and 297 mμ.

SPECTROPHOTOMETRIC DETERMINATION OF CAFFEINE IN AQUEOUS SOLUTION—

Evaporate a 5- or 10-ml aliquot of the chloroform solution on a steam-bath, dissolve the residue in water, transfer the solution to a 50-ml calibrated flask, and dilute to the mark. Measure the optical density of this solution at 250, 273 and 296 mμ, and hence calculate the caffeine content of the sample.

DETERMINATION OF NITROGEN CONTENT OF CHLOROFORM EXTRACT—

Concentrate the extract to less than 10 ml by evaporation or distillation. In the digestion flask place approximately 1·50 g of potassium sulphate and 40 mg of mercuric oxide, add a few beads to prevent "bumping" and rinse the neck of the flask with 3 ml of chloroform. Place the flask on a digestion rack, and continue as described by the A.O.A.C.[8]; use 0·01 N acid, 1 ml of which is equivalent to 0·485 mg of caffeine.

CALCULATION—

The values of $E_{1cm}^{0·001\%}$ for a solution of pure caffeine are determined at the wavelengths of maximum absorption and of points on the ascending and descending portions of the curve equidistant from the maximum. Let these values be M, A and D, respectively, and let the corresponding values for the test solution at the same wavelengths be M′, A′ and D′. The wavelengths at which the absorption of caffeine is measured in chloroform and in aqueous solution are shown below.

	A	M	D
Wavelength for chloroform solution, mμ ..	257	277	297
Wavelength for aqueous solution, mμ ..	250	273	296

The percentage of caffeine in the sample (P) can be calculated from the equation—

$$P = \frac{M' - \frac{1}{2}(A' + D')}{M - \frac{1}{2}(A + D)} \times \frac{200}{v} \times \frac{50}{0·001\,w}$$

in which 200 ml is the total volume of chloroform solution collected from the column, v ml is the aliquot of the chloroform extract used, 50 ml is the volume to which the aliquot is diluted and w is the weight of sample used (in grams).

When a Hilger spectrophotometer and 1-cm cells are used, the values of $[M - \frac{1}{2}(A + D)]$ for a 0·001 per cent. solution of caffeine in chloroform and in water are, respectively, 0·430 and 0·415. These factors can be used in routine determinations, but for more accurate work they should be found independently in order to allow for differences between instruments, in absorption characteristics and, particularly, between the light paths of cells.

TABLE II

COMPARISON OF RESULTS

Sample No.	Number of determinations by each method	Caffeine content found by— spectrophotometry with background correction— in chloroform, %	spectrophotometry with background correction— in water, %	calculation from nitrogen content, %	Bailey - Andrew method, %
Roasted coffee—					
1	10	1·59	1·60	1·70	1·60
2	10	1·41	1·48	1·52	1·45
3	3	1·88	1·92	1·96	1·87
4	3	1·32	1·30	1·40	1·34
Coffee mixed with maize—					
1	10	1·02	1·07	1·12	1·00
2	10	0·89	0·88	0·93	0·88
3	3	0·71	0·73	0·81	0·71
4	3	0·92	0·94	0·99	0·89
Coffee mixed with chicory—					
1	12	—	—	1·12	1·08
2	5	—	—	1·15	1·12
Soluble coffee—					
1	4	3·49	3·43	3·62	3·53

Comparison of methods

Samples of each type were analysed by the proposed method, by determining the nitrogen content of the chloroform eluate and by the modified Bailey - Andrew procedure[7]; the results are shown in Table II. It can be seen that the proposed method gives results for both chloroform and aqueous solutions in close agreement with those found by the modified Bailey - Andrew method. Results for caffeine calculated from the nitrogen contents of the chloroform eluates are generally slightly high, but this procedure can be used, if necessary, for checking results by the proposed method. It is particularly useful for samples containing chicory, to which the spectrophotometric method cannot be applied, and results so obtained for such samples are in excellent agreement with those found by the modified Bailey - Andrew procedure. The nitrogen contents of several samples of chicory and maize extracts were determined, and it was found that the "caffeine" contents of these samples, as calculated from the nitrogen values, were not greater than 0·06 mg per g.

To ascertain the effectiveness of the extraction procedure, known amounts of caffeine were added to some samples, and the recoveries are shown in Table III.

After the completion of this work, my attention was directed to a paper on the spectrophotometric determination of caffeine in roasted coffee,[9] in which the same extraction procedures were used, but without background correction.

Table III

Recovery of caffeine from coffee by proposed method

Sample No.	Weight of sample, g	Caffeine found, mg	Caffeine content found by— proposed method (chloroform extract), %	Caffeine content found by— Bailey - Andrew method, %
Roasted coffee—				
1	1·009	15·8	1·58	1·55
	1·009 + 5 mg of caffeine	20·9	1·59	—
Coffee mixed with maize—				
1	1·000	10·3	1·03	1·00
	1·008 + 5 mg of caffeine	15·2	1·02	—

Conclusions

The proposed method fulfils the requirements of a routine method for determining caffeine in coffee. The extraction and dilution procedures are simple and require no special technique or constant attention. The method does not involve use of any special reagent, nor does it include tedious filtration or extraction procedures. Unlike methods involving oxidation of interfering substances by permanganate,[3] critical control of pH is not necessary. The method was applicable to all types of coffee studied, except those containing chicory, for which the chloroform extract is evaporated and the amount of caffeine present determined from its nitrogen content. Results by the proposed method are in good agreement with those obtained by the official procedure, and one analyst can make six to eight assays in an 8-hour day.

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An Improved Procedure for the Direct Spectrographic Analysis of Plant Ash

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A method is decribed for the spectrographic analysis of plant ash; air from a jet of the type described by Stallwood is blown around the arc to intensify the weak zinc, boron and phosphorus lines and at the same time to decrease the intensity of the other strong lines. By this method, it is possible to determine calcium, magnesium, sodium, phosphorus, copper, zinc, boron, iron, aluminium, manganese, barium, nickel and chromium directly on the ash with one exposure and without sacrifice of accuracy.

THERE are important advantages to be gained from direct spectrographic determinations on plant ash, as methods of concentration are time-consuming and liable to errors caused by contamination or loss. Further, spectrographic determinations of a single element in plant ash are seldom worth-while, as chemical methods are often more suitable. It is obviously desirable to determine as many elements as possible simultaneously in a single exposure. However, with the procedures generally used in agricultural laboratories, it is unfortunately not possible to determine zinc, boron and phosphorus (together with the more easily determined constituents) directly on the ash. The method described here was evolved in an attempt to determine these three elements simultaneously with the others and so to obtain the maximum amount of information from the direct examination of the ash.

Mitchell and Scott's concentration method[1] serves for determining a wide range of trace elements in plant materials, but does not give results for potassium, calcium, magnesium, sodium, barium, strontium, manganese, boron and phosphorus. Farmer's method,[2] in which potassium sulphate is used as a spectroscopic buffer in the direct examination of the ash, has been applied in this laboratory and, with slight modification, has given satisfactory results for calcium, magnesium, sodium, copper, manganese, iron and aluminium; nickel and chromium can also be included if present in toxic amounts. Satisfactory lines are obtained with barium and strontium, but results for zinc, boron and phosphorus are of little use, as, with most plant materials, the lines are too weak. Many methods can be used to intensify these lines, *e.g.*, increasing the amount of ash in the sample electrode, widening the slit, increasing the current, and so on, but all these methods increase the intensities of the copper and other strong lines, thereby making them unreadable on a micro-photometer. The only means by which the zinc line can be preferentially intensified are—

(*i*) by using special quartz lenses having a high transmission around 2000 A,

(*ii*) by using specially treated photographic plates having a high sensitivity in the region of the zinc line and

(*iii*) by blowing air around the arc, so removing the cooler vapour, which re-absorbs the radiation from zinc.

Several methods have been proposed for determining zinc spectrographically, either directly on the ash[3 to 7] or after preliminary separation and concentration.[1,8] Strasheim and Eve[7] showed that the intensity of the zinc line and the slope of the working curve were improved by blowing air around the arc. This method has been developed and combined with Farmer's method, so that all the elements measured by Farmer's method, together with zinc, phosphorus and boron, can be determined in one exposure. Further, Farmer's method is only applicable at low levels of sodium, and, when significant amounts of sodium are found, a second determination must be carried out on the diluted ash; the proposed method covers a more useful range of levels of sodium.

The important elements that can be determined spectrographically in plant ash, but are not determined by the proposed method, are potassium, molybdenum and cobalt. Potassium can be conveniently determined by flame photometry, but preliminary separation and concentration is necessary for determining molybdenum and cobalt.

Experimental

Apparatus—

Spectrograph—Hilger medium-quartz.

Source unit—A 200-volt d.c. generator (maximum current 30 amps), with a variable resistor to permit the current to be kept constant at 9 amps.

External optics—Immediately in front of the slit is placed a 7-division step-sector and an F1026 quartz lens, which throws an image of the arc on to the collimating lens. A screen at this point allows light from the central 4-mm portion of the arc to pass on.

Plates—Kodak B10.

Micro-photometer—A Hilger non-recording micro-photometer was used, with the density-curve separation method and without background correction.

Excitation conditions—

The standard excitation conditions are described below.

Current—9 amps.

Upper electrode (cathode)—4·7 mm; boron-free carbon.

Sample electrode (anode)—5·5 mm (7/32 inch); carbon, with crater 7 mm deep and 5/32 inch in diameter. The sample electrode is slightly thicker than the upper electrode because a larger upper electrode projects too far into the air stream and leads to irregular burning. For this reason, it is necessary to align the electrodes as acccurately as possible before beginning the burn.

Jet—A jet of the type described by Stallwood,[9] 8 mm in diameter, with the width of the annular orifice 0·25 mm (0·01 inch) and an air pressure of 10 inches of oil. The jet was constructed by fitting a tube 8 mm in external diameter into a hollow truncated cone having a hole 8·5 mm in diameter in its top, so producing a gap of 0·25 mm around the central tube, which projected 2·5 mm above the cone to decrease the tendency of the stream of air to converge; the whole unit was made of brass and silver plated. A small compressor was used as a source of air pressure, and a 1-litre filter-flask was fitted in the air line to smooth out the pulses from the compressor. The air pressure, which increased slightly during the burn, was kept constant by providing an escape tube dipping 10 inches below the surface of a column of oil.

Electrode gap—A gap of 8 mm was maintained throughout the exposure, but only the central 4-mm portion was photographed.

Buffer mixture—Experience confirmed Farmer's statement that of all the salts of potassium and sodium tested, potassium sulphate is the most promising material for use as a buffer, as it contains no water of crystallisation, burns steadily in the arc and is considerably less volatile than the other salts tried. Farmer's mixture was used, *i.e.*, 2 parts of carbon and 1 part each of potassium sulphate and ash.

Internal standards—

For copper, iron, manganese, nickel and chromium—Mixture containing 0·1 per cent. of tin, as stannic oxide, in carbon.

For calcium, magnesium and sodium—Mixture containing 0·1 per cent. of lithium, as sulphate, in carbon.

For boron and aluminium—Mixture containing 0·1 per cent. of beryllium, as oxide, in carbon.

For zinc and phosphorus—Mixture containing 0·5 per cent. of tellurium, as oxide, in carbon.

These amounts of internal standards were chosen to give, as far as possible, lines of approximately the same density as those of the elements being measured at deficiency or toxicity level, whichever might apply. This decreased the necessity for background correction to a minimum.

Standard samples—

Prepared mixtures were used.

Method

To avoid errors caused by moisture and by grinding small amounts of material in an agate mortar, the procedure described below is recommended.

PROCEDURE—

Ash the sample at 420° C, weigh the ash, and place it in a sample tube that has been well dried, together with its cork, on a hot-plate. If the original specimen has been adequately cleaned, the ash can be reduced to a fine powder and homogenised by stirring with a glass rod. Reject all but about 200 mg of the ash, and place the specimen tubes on a hot-plate to remove the moisture absorbed in the weighing process. (The ash will not be dried satisfactorily if more than about 200 mg of it are present in the sample tube.)

Weigh 30 mg of ash and 90 mg of finely ground carbon mixed with potassium sulphate and internal standards into a small clear Vitreosil crucible that has been dried on a hot-plate; eight exposures can be made on one plate, so that eight crucibles are necessary. Mix the powders by stirring with a clear Vitreosil rod. (Clear Vitreosil contains 99·99 per cent. of silica, so that the possibility of contamination is small.) Keep the crucibles warm on the hot-plate until the electrodes are ready for packing; by this means, the powders are kept as dry as possible and the grinding of small amounts of powder is avoided. After they have been packed, heat the electrodes at 200° C for at least 1 hour, otherwise an irregular burn may result from moisture in the ash.

When placing a filled electrode in the stand, take care to ensure that its position relative to the jet is always the same; the top of the electrode should be 1 inch above the orifice of the jet. The burn takes from 3 minutes 15 seconds to 3 minutes 25 seconds, depending on the nature of the ash. If the burn is allowed to proceed to the end, the current of air breaks the arc; about 4 seconds before this happens, the current starts to decrease rapidly, and this point is taken as being the end of the burn.

RESULTS

Table I shows the lines used, the range of concentrations covered in the plant ash and the reproducibility of the results obtained.

TABLE I

CONCENTRATIONS OF ELEMENTS DETERMINABLE IN PLANT ASH

Element	Analytical line, A	Internal standard line, A	Range of concentrations determinable	Reproducibility (in terms of standard deviation),* %
Calcium	3180·5	Li 3232·6	1 to 20 per cent.	4·2
	3158·9		0·1 to 1 per cent.	
Magnesium	3093·0		0·8 to 8 per cent.	4·7
Sodium	3302·2		0·20 to >7 per cent.	2·5
Phosphorus	2136·2	Te 2142·8	0·1 to 3·5 per cent.	3·2
Copper	3274·0	Sn 3262·3	10 to 3000 p.p.m.	2·2
	3247·5		1 to 1000 p.p.m.	
Zinc	2138·6	Te 2142·8	3 to 3000 p.p.m.	5·0
Iron	2719·0	Sn 2706·5	100 to 10,000 p.p.m.	4·4
Aluminium	3082·2	Be 3321·3	60 to 10,000 p.p.m.	6·1
Manganese	2801·1†	Sn 2840·0	60 to 10,000 p.p.m.	2·3
	2593·7	Sn 2706·5	100 to 10,000 p.p.m.	
Boron	2497·7	Be 2494·7	30 to 1000 p.p.m.	10·9

* These figures refer to the means of duplicate determinations on eight pairs of sub-samples from the same original sample of ash.

† With samples relatively high in magnesium and low in manganese, *e.g.*, maize-meal ash, there is interference between the manganese line at 2801·1 A and the intense magnesium line at 2802·7 A; for such samples, the manganese line at 2593·7 A must be used.

DISCUSSION OF THE METHOD

EFFECTS OF AIR BLOWN AROUND THE ARC—

It was evident from Strasheim and Eve's paper[7] that the intensity of the zinc line at 2138·6 A could be improved by blowing air around the arc with a jet of the type described by Stallwood.[9] With the jet described above, studies were made of the effect of variation in air pressure in the jet on the line intensities produced. The intensities of the zinc, boron and phosphorus lines increased with increasing air pressure up to a certain point and then

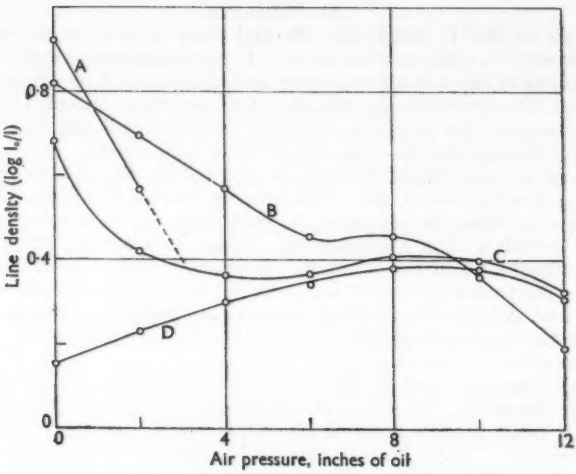

Fig. 1. Effect of air pressure on line intensity: curve A, sodium line at 3302 A (0·05 per cent. of sodium in ash); curve B, calcium line at 2997 A (3·2 per cent. of calcium in ash); curve C, lithium line at 3232 A (0·2 per cent. of lithium in ash); curve D, zinc line at 2138 A (190 p.p.m. of zinc in ash)

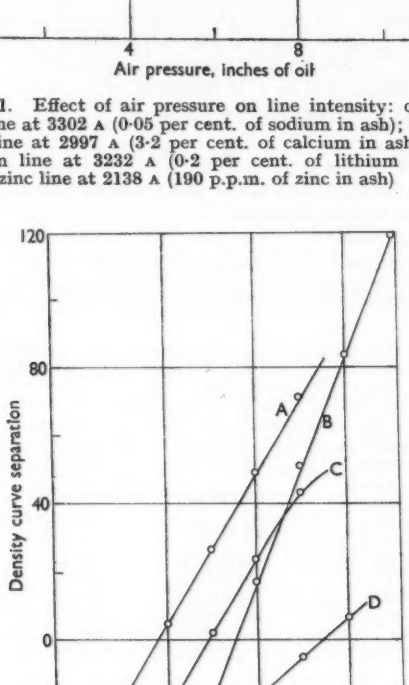

Fig. 2. Working curves: curve A, line pair Zn 2138 - Te 2142, with draught; curve B, line pair Fe 2719 - Sn 2706, with draught; curve C, line pair Fe 2719 - Sn 2706, without draught; curve D, line pair Zn 2138 - Sn 2429, without draught

decreased. At the same time, the line intensities for the more easily ionisable elements (such as calcium, magnesium and copper) were at first decreased; further increase in air pressure produced a slight increase in intensity, and, finally, a decrease was produced at the same pressure, as was found for the zinc and boron lines. Typical effects on selected lines are shown in Fig. 1.

The point at which all lines are decreased in intensity is the point at which nearly the whole of the luminous envelope of the arc is removed and all that remains is the central column of the arc between the electrodes. An increase in air pressure beyond this point cuts into the arc column itself and causes an all-round decrease in line intensity. The air pressure required to produce this effect depends on the dimensions of the jet; with a wider orifice, the effect takes place at a lower pressure.

The increase in intensity of the zinc line permits a 10-fold decrease in the minimum amount of zinc detectable. As the more intense lines are decreased in intensity, it is possible to increase the amount of ash used in the sample electrode by a factor of 3 and still to obtain readable lines at the higher concentrations. In this way, sensitivity for zinc can be increased by a factor of about 30. At the same time, a good phosphorus line is obtained, and the intensity of the boron line is considerably increased; there are also several other advantages that should result in improved accuracy.

The slopes of the working curves are increased by 25 to 80 per cent., *i.e.*, a given increase in concentration produces an increase in line intensity greater than that produced without draught (see Fig. 2). Although this increase in slope gives greater accuracy, it also tends to decrease the range of concentrations over which readings can be taken, but this effect is compensated for by the fact that self-absorption of the lines is, for most elements, eliminated.

The effect of variations in matrix composition is decreased. With Farmer's method,[2] the line intensities for sodium, magnesium and calcium are affected by high concentrations of silicon in the ash, and a similar effect occurs for sodium and magnesium with ashes high in calcium. With air blowing around the arc, the corresponding matrix effects on sodium and magnesium are negligible, and the effect on calcium is much decreased. It is necessary, however, to prepare several working curves for phosphorus from standard samples having different contents of silicon, as a high proportion of silicon in the ash depresses the line appreciably.

Although the background in the shorter-wavelength region is slightly increased, that in the longer-wavelength region is considerably decreased. Wandering of the arc is eliminated, and the end-point of the burn is clearly defined. The air stream has a strong cooling effect on the sample electrode, and this would be expected to decrease fractional volatilisation; that this is so is evident from watching the burn. With the proportion of buffer used, when an ash low in potassium is burnt without draught round the arc there is a tendency for the potassium to disappear before the end of the burn and for the character of the arc to change. This tendency is eliminated by blowing air around the arc. Larger electrodes are easier to pack satisfactorily, and there is less chance of error caused by inadequate mixing of the ash when larger amounts are used. An unexpected advantage found was the decrease in the intensity of copper lines in the blanks and hence a lower limit of determination for copper. Most air compressors produce a certain amount of oil spray; this was trapped in the 1-litre filter-flask fitted to smooth out the pulses from the compressor. Incoming air impinged on the oil in the bottom of the flask, and this acted as an excellent air-cleaning arrangement, so that the arc was surrounded by dust-free air and contamination of the arc by aerial dust was much decreased.

The only disadvantage of the proposed method is that the electrodes burn away more rapidly, and about 50 per cent. more carbon rod is used for each sample than is used in Farmer's method. This relatively rapid burning of the carbon rod necessitates frequent adjustment to keep the length of the arc constant. Further, the electrical resistance of the arc is markedly affected by its length, and it is more difficult to keep the arc current constant. However, with some practice, reasonably constant conditions can be attained.

ASHING PROCEDURE—

The temperature of ashing is kept as low as possible to minimise damage to the silica dishes. As shown by Gorsuch,[10] no loss of zinc takes place up to 900° C, and the possibility of volatilising zinc at temperatures in excess of 450° C is therefore not a consideration in determining ashing temperature. Gorsuch found that considerable loss of copper occurred

during dry ashing, and this was attributed to some physical or chemical reaction between copper and the silica of the basin. Comrie,[11] however, found that loss of copper was eliminated by adding magnesium nitrate to produce a bulky ash and removing the ash from the silica dish before solution in acid. It seems probable that the loss of copper found by Gorsuch took place when acid was added to the ash in the silica dish. It is therefore considered that, as no acid is added to the ash in the proposed procedure, loss owing to retention of copper on the silica dish will not be serious.

It is generally considered that loss of phosphorus during dry ashing of plant material is small, except for materials (such as seeds) producing an ash having a low base content or a high phosphorus content. As the technique described will only give results up to 3·5 per cent. of phosphorus in the ash, the phosphorus contents of such materials cannot be determined by the proposed method, which was developed mainly for foliar analysis.

PREPARATION OF ASH FOR THE ARC—

Potassium carbonate, which is present in all plant ash, is an extremely hygroscopic substance, and moisture in the ash has been found to affect the mixing as well as the burning of the ash mixture. Improved burns are obtained when the procedure previously described is adopted; this keeps the ash as dry as possible at all stages of preparation of the filled electrode. A second source of error, which is not generally recognised, arises from grinding small amounts of powder in an agate mortar. When mixtures of salts resembling plant ash were ground in a mortar, some of the material adhered to the mortar in a thin layer. This layer was not representative of the original sample and consisted mainly of calcium carbonate; the ratio of calcium to copper in the residual material was about 10 times that in the original material. When plant ash was ground in a mortar, the residual material was mainly calcium and aluminium. Results for chromium in ashes high in calcium were particularly unreliable because of this effect. A series of mixtures resembling plant ash was prepared, containing 100 p.p.m. of chromium, but having steadily increasing calcium contents. Carbon and potassium sulphate were added to the mixtures, which were then ground in a mortar and analysed spectrographically. For calcium contents up to 6·5 per cent., the effect on the line intensity for chromium was small, but at 10 per cent. of calcium the chromium line practically disappeared. When the chromium was mixed with carbon first and then mixed with the prepared ash, the effect was much less. Further, a 50 per cent. decrease in the apparent-chromium content of an ash high in calcium could be produced merely by grinding a small amount of the ash in an agate mortar. It is possible that similar difficulties are encountered with boron and potassium sulphate, and other determinations may be affected.

I thank Dr. D. H. Saunder for helpful advice and discussion.

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Colorimetric Determination of Tin in Propellants by Means of Haematoxylin

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A method is proposed for the colorimetric determination of metallic tin in propellants by the use of haematoxylin. A 0·3-g sample is treated with a mixture of nitric, sulphuric and perchloric acids, and the solution is evaporated until fumes of sulphur trioxide are evolved. To an aliquot of this solution are added haematoxylin, gelatin and sodium acetate, and the solution is heated for 15 minutes in boiling water. The factors influencing development of colour have been studied in detail. The method of developing the colour is probably applicable to the determination of other metals as well as tin.

Metallic tin in propellants has been determined iodimetrically or polarographically after addition of sulphuric and nitric acids, evaporation to fumes of sulphur trioxide and further treatment with nitric acid.[1] Metallic tin and tin dioxide in propellants have been determined by digestion with nitric acid, filtration, ignition and correction for impurities.[1,2]

These methods are somewhat tedious, and this laboratory undertook the development of a rapid colorimetric method. Haematoxylin was selected as the reagent, since it seemed to be one of the few reagents for the colorimetric determination of tin that could be used in presence of lead, an element frequently found in propellants.

The use of haematoxylin for the colorimetric determination of tin seems to have been first proposed by Tartakovskii,[3] who made the solution slightly alkaline with sodium hydroxide, added haematoxylin and compared the red colour produced with standards; a solution of stannous tin was used, but some oxidation probably took place during development of colour. The method was unreliable, as haematoxylin itself produces a red colour at pH values greater than 8 (for this reason, it finds some application as an indicator[4]). Charlot[5] investigated the use of haematoxylin as a qualitative reagent for tin and other metals. Teicher and Gordon[6] developed colour at pH 0·8, but the pink colour produced was not sensitive and did not obey Beer's law. Kojima[7] used oxidised haematoxylin and developed colour in a solution containing 2 drops of 10 per cent. sulphuric acid per 5 ml.

In this paper, an improved method is proposed for the colorimetric determination of tin with haematoxylin; the solution is treated with haematoxylin, gelatin and sodium acetate and then heated in boiling water for 15 minutes. The method is much more sensitive than were previous methods involving use of haematoxylin, and the colour obeys Beer's law. The colour appears to be brown in presence of less than 0·04 mg of tin and amber for 0·04 to 0·1 mg, but the true colour is somewhat masked by the yellow of the blank. The method has been applied to ball propellants after treating the samples with a mixture of nitric, sulphuric and perchloric acids and evaporating to fumes of sulphur trioxide.

Experimental

Effect of amount of gelatin—

The coloured lake produced with haematoxylin was precipitated unless gelatin was present, and experiments were carried out to ascertain how much gelatin was desirable. Aliquots (5 ml) of standard tin solution B (see "Reagents," p. 839), *i.e.*, 0·060 mg of tin and 0·2 ml of sulphuric acid, were placed, by pipette, in 50-ml calibrated flasks, and 15 ml of water, 5 ml of 0·1 per cent. haematoxylin solution, a known volume of 1 per cent. gelatin solution and 10 ml of 50 per cent. sodium acetate solution were added to the contents of each flask. The solutions were heated in boiling water for 13 minutes and cooled, and each was then diluted to the mark. The transmission was measured at 585 mμ with a Beckman model B spectrophotometer that had been set to give 100 per cent. transmission with water. The results are shown in Fig. 1, from which it can be seen that the amount of gelatin present was not too critical; the use of 4 ml of 1 per cent. gelatin solution was adopted.

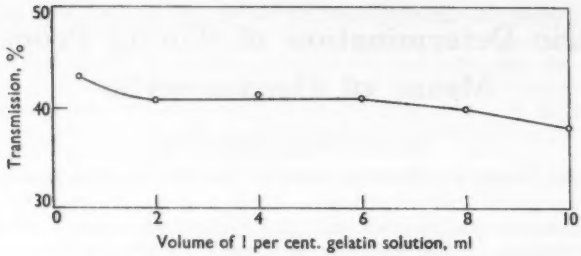

Fig. 1. Effect of amount of gelatin on colour

EFFECT OF AMOUNT OF HAEMATOXYLIN—

The effect of the amount of haematoxylin solution used was investigated in experiments of the type described above for gelatin. The amount of haematoxylin was somewhat critical, as shown in Fig. 2; the use of 4 ml of the reagent solution, measured accurately, was adopted. It was essential to use a solution of haematoxylin in 95 per cent. ethanol containing 1 drop of hydrochloric acid per 100 ml, as recommended by Teicher and Gordon[6]; without the hydrochloric acid, the solution darkened rapidly and results were erratic. Further, it is recommended that the haematoxylin solution be prepared freshly each day. The blank value tended to increase as the haematoxylin solution aged from 10 hours to 1 week; however, during the same period, the intensity of the colour obtained for the tin increased somewhat (even after setting the spectrophotometer to give 100 per cent. transmission with the blank). The blank value became steadier as the haematoxylin solution aged from 1 to 2 weeks, but, at the same time, the intensity of the tin colour decreased somewhat. The haematoxylin solution can be used for up to 8 days if the calibration graph is prepared on the same day that the sample is analysed or if a standard is analysed at the same time as the sample. It was not necessary to filter the haematoxylin solution, as a clear solution was always obtained.

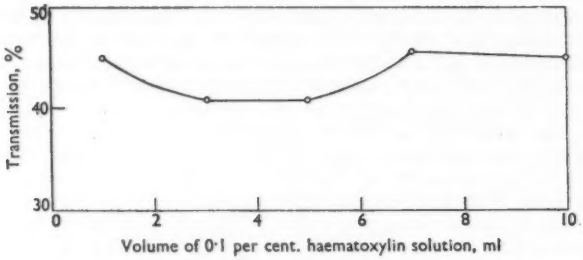

Fig. 2. Effect of amount of haematoxylin on colour

EFFECT OF INITIAL ACIDITY—

Ten millilitres of sulphuric acid was the optimum amount that should be present in the 250 ml of solution from which the 5-ml aliquot was taken for development of colour. The presence of more than 10 ml of this acid made it difficult to buffer the solution with sodium acetate, and the presence of less could cause hydrolysis of the tin. The normality of the solution in the 250-ml flask was about 1·4; the normality of the solution after the 5-ml aliquot had been taken and water, haematoxylin solution and gelatin had been added was about 0·26.

EFFECT OF AMOUNT OF SODIUM ACETATE SOLUTION—

The intensity of colour and the pH increased with increasing amounts of 50 per cent. sodium acetate solution, but levelled out when about 10 ml were present (see Figs. 3 and 4). The intensity of the yellow colour of the blank increased with increasing amounts of sodium acetate, as shown in Fig. 5. From a consideration of Figs. 3, 4 and 5, the use of 10 ml of 50 per cent. sodium acetate solution, which gave a pH of 5·4, seemed desirable. Results

when the 10 ml of sodium acetate solution were added in one portion from a graduated cylinder were better than those obtained when the addition was made by pipette.

DEVELOPMENT OF COLOUR—

Effect of volume—It is recommended that 15 ml of water be added to the 5-ml aliquot of sample, so that the volume is about 38 ml when the solution is heated. Results were low when the 15 ml of water were omitted.

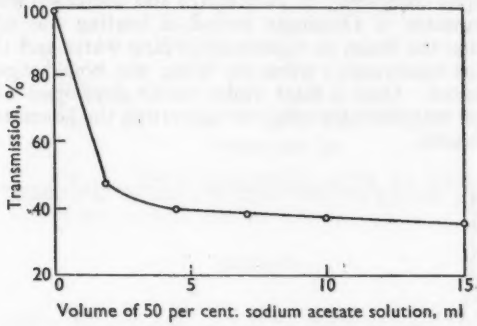

Fig. 3. Effect of amount of sodium acetate on colour

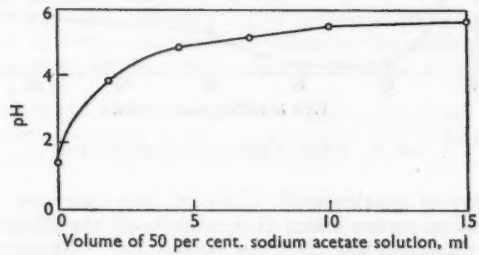

Fig. 4. Effect of amount of sodium acetate on pH

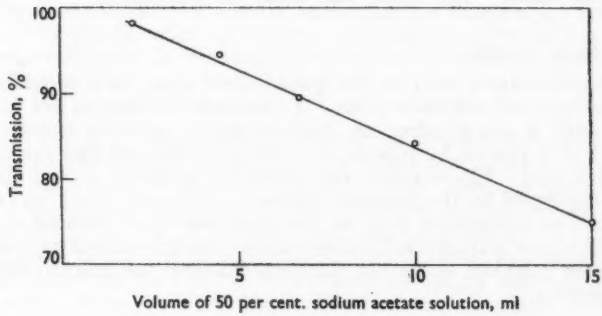

Fig. 5. Effect of amount of sodium acetate on blank

Effect of order of adding reagents—Satisfactory results were obtained when the reagents were added in the order haematoxylin, gelatin and sodium acetate or haematoxylin, sodium acetate and gelatin or gelatin, haematoxylin and sodium acetate. Some of the coloured lake was precipitated when the reagents were added in the order sodium acetate, haematoxylin and gelatin or gelatin, sodium acetate and haematoxylin; it therefore appears that precipitation occurs when the sodium acetate is added before the haematoxylin.

Effect of time between addition of haematoxylin and sodium acetate solution—Since the faint red colour produced by the reaction of tin with haematoxylin in slightly acid solution requires up to 40 minutes for full development,[6] it seemed advisable to ascertain whether or not this colour had to be completely developed before the sodium acetate solution was added. Experiments showed that results were the same whether the interval between the addition of the haematoxylin and sodium acetate solutions was 10 seconds or 4 hours.

Effect of time of heating—The effect of time of heating in boiling water is shown in Fig. 6. The colour was developed completely in 12 minutes and decreased when the time of heating was longer than 20 minutes; a 15-minute period of heating was adopted. It was found to be preferable to place the flasks in vigorously boiling water and then to lower the flame so that the water boiled moderately; when the water was boiled vigorously for 15 minutes, results were slightly lower. Only a faint violet colour developed when the solutions were not boiled. The boiling may have the effect of converting the haematoxylin to a degradation product (possibly haematin).

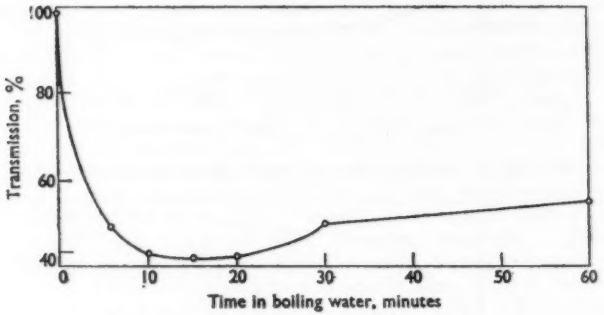

Fig. 6. Effect of time of boiling on colour

Effect of temperature of measurement—When the solutions were in boiling water, they seemed to have more of an amber colour than when cool; the effect of the temperature at which the colour was measured was therefore investigated. Readings were the same over the range 17° to 30° C.

Stability of the colour—The colour was stable for 8 hours.

EFFECT OF VALENCY OF TIN—

In the proposed method, tin is in the quadrivalent state, as it is oxidised by treatment with nitric, perchloric and sulphuric acids. To find out whether or not bivalent tin would give the same result as quadrivalent tin, 3 ml of freshly prepared standard tin solution A (see "Reagents") were placed, by pipette, in a 250-ml calibrated flask containing water and 9·8 ml of sulphuric acid. The solution was diluted to the mark, a 5-ml aliquot was taken, and colour was developed by the proposed method. The same result was obtained as with quadrivalent tin. The explanation may be that stannous tin is oxidised during the boiling. There is no danger of loss of tin when tin solutions containing hydrochloric acid are evaporated to fumes of sulphur trioxide, as neither stannous chloride[8] nor stannic chloride[9] is volatile in these circumstances.

SPECTROPHOTOMETRIC CURVES—

The spectrophotometric curves for the coloured and blank solutions are shown in Fig. 7; water was used as reference solution. There is a plateau for the transmission of the colour extending from about 540 to 560 mμ, and maximum absorption probably occurs at about 550 mμ. The colour of the blank increases over the length of the plateau region. From a consideration of Fig. 7, it seemed most suitable to measure the transmission at 590 mμ to minimise the effect of the blank.

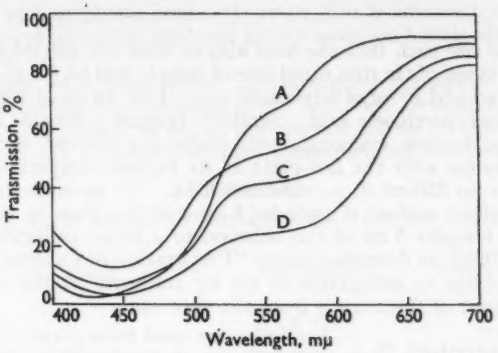

Fig. 7. Spectrophotometric curves for different amounts of tin: curve A, blank; curve B, 0·04 mg; curve C, 0·06 mg; curve D, 0·10 mg

Method

Reagents—

Haematoxylin solution, 0·1 per cent.—Dissolve 0·1 g of haematoxylin in 80 ml of 95 per cent. ethanol, add 1 drop of hydrochloric acid, sp.gr. 1·19, and dilute to 100 ml with the ethanol. Prepare this solution freshly each day.

Gelatin solution, 1 per cent.—Dissolve 2 g of gelatin in 200 ml of water by warming at about 50° C, and cool to room temperature. Prepare this solution freshly every 2 days.

Sodium acetate solution, 50 per cent.—Dissolve 500 g of sodium acetate trihydrate in water, and dilute to 1 litre.

Standard tin solution A—Dissolve 1·0000 g of pure granulated tin in 100 ml of hydrochloric acid, sp.gr. 1·19, by heating at 40° C in a covered 400-ml beaker. Add about 300 ml of water, cool, and dilute to 1 litre in a calibrated flask.

$$1 \text{ ml} \equiv 1{\cdot}00 \text{ mg of tin.}$$

Standard tin solution B—Measure 3·0 ml of standard tin solution A into a 250-ml beaker, and add 10 ml each of nitric acid, sp.gr. 1·42, and sulphuric acid, sp.gr. 1·84, and 1 ml of 70 per cent. perchloric acid. Cover with a watch-glass, evaporate until fumes are evolved, and then, with the cover ajar, heat for 12 minutes with the hot-plate at its highest temperature. (According to the manufacturer's literature, the maximum temperature attainable with ours is about 275° C.) Cool, add water, cool again, and dilute to 250 ml in a calibrated flask.

Blank solution—Prepare as described for standard tin solution B, but omit the tin.

Preparation of calibration graph—

Measure 1·0-, 2·0-, 3·0-, 4·0- and 5·0-ml portions of standard tin solution A into separate 250-ml beakers, and add to each portion 10 ml of nitric acid, sp.gr. 1·42, 10 ml of sulphuric acid, sp.gr. 1·84, and 1 ml of 70 per cent. perchloric acid. Similarly prepare a reagent blank solution. Cover the beakers with watch-glasses, evaporate until fumes are evolved, and then, with the covers ajar, heat for 12 minutes with the hot-plate at its highest temperature. Cool, add water, cool again, and dilute each solution to 250 ml in a calibrated flask.

By pipette, place 5-ml portions of these solution in 50-ml calibrated flasks, and add to each 15 ml of water, 4·0 ml of haematoxylin solution, 4 ml of gelatin solution and 10 ml of sodium acetate solution. (Measure the haematoxylin solution with a pipette or burette and the other reagents with a graduated cylinder.) Place the flasks in 400-ml beakers containing 300 ml of vigorously boiling tap-water, lower the flame so that the water boils moderately, and keep the flasks in the water for 15 minutes. Remove the flasks, cool to room temperature in a water bath, dilute each solution to the mark, and measure its transmission at 590 mμ with a spectrophotometer that has been set to give 100 per cent. transmission with the reagent blank. Plot a graph of tin present in the 50-ml solution (in milligrams) against percentage transmission; graphs so prepared are linear, *i.e.*, the colour obeys Beer's law.

PROCEDURE—

Take a sample of size such that the 5-ml aliquot from the 250 ml of solution will contain preferably 0·05 to 0·08 mg of tin (the usual size of sample will be 0·3 g). Transfer the sample to a 250-ml beaker, and add 10 ml of nitric acid, sp.gr. 1·42, 10 ml of sulphuric acid, sp.gr. 1·84, and 1 ml of 70 per cent. perchloric acid. Similarly prepare a reagent blank solution. Cover the beaker with a watch-glass, evaporate until fumes are evolved, and then, with the cover ajar, heat for 12 minutes with the hot-plate at its highest temperature. Cool, add water, cool again, and dilute to 250-ml in a calibrated flask. (If barium, strontium, lead or silica is present, shake the flask, and set it aside for 1 hour at this stage so that the precipitate will settle.) By pipette, transfer 5 ml of this solution to a 50-ml calibrated flask, add 15 ml of water, and develop colour as described under "Preparation of Calibration Graph." Convert the transmission readings to milligrams of tin by reference to the calibration graph, and calculate the tin content of the sample from the expression—

$$\text{Tin content, \%} = \frac{\text{Amount of tin read from graph, mg}}{\text{Weight of sample in aliquot, g} \times 10}$$

Note that the calibrated flasks in which the colour is developed and the cells of the spectrophotometer must be rinsed with hydrochloric acid before being used for the next determination.

INTERFERENCES

To test the possible effects of interfering cations, 1-mg amounts of various metals were added to 5-ml portions of blank solution, and these samples were subjected to the proposed procedure; for elements that did not interfere, further tests were made in the presence of tin. The results are shown in Tables I and II. It can be seen from Table I that thirteen elements

TABLE I

METALS INTERFERING WITH COLORIMETRIC DETERMINATION OF TIN

Element tested	Interference in absence of tin	Element tested	Interference in absence of tin
Aluminium, as $AlCl_3$	Violet lake	Titanium, as $TiCl_4$	Violet lake
Antimony, as $SbCl_3$	Violet lake	Tungsten, as Na_2WO_4	Violet lake
Bismuth, as $BiONO_3$	Violet lake	Vanadium, as $(VO)_2Cl$	Red-violet lake
Chromium, as $Cr(NO_3)_3$	Blue-violet lake	Zirconium, as $Zr(NO_3)_4$	Violet lake
Copper, as $CuSO_4$	Red-violet lake	Cerium, as $Ce(SO_4)_2$	Orange colour
Germanium, as GeO_2	Violet lake	Mercury, as $HgCl_2$	Turbid brown colour
Iron, as $Fe_2(SO_4)_3$	Violet lake	Selenium, as H_2SeO_3	Brown colour
Molybdenum, as $(NH_4)_2MoO_4$	Blue-violet lake	Uranium, as $UO_2(C_2H_3O_2)_2$	Orange colour
Thorium, as $Th(NO_3)_4$	Red-violet lake	Silver, as $AgNO_3$	Silver chloride and elemental silver precipitated

interfered by producing lakes, four by producing colours of not too great an intensity that were apparently not lakes and silver by producing a precipitate of silver chloride in the cold and elemental silver on boiling. Table II shows that sixteen elements did not interfere.

TABLE II

RECOVERY OF TIN IN PRESENCE OF VARIOUS METALS

Element tested	Recovery of 0·06 mg of tin, mg	Element tested	Recovery of 0·06 mg of tin, mg
Arsenic, as As_2O_3	0·060	Lithium, as LiCl	0·059
Barium, as $BaCl_2$	0·058	Magnesium, as $MgSO_4$ ..	0·060
Beryllium, as $BeSO_4$	0·060	Manganese, as $MnSO_4$	0·062
Boron, as H_3BO_3	0·059	Nickel, as $NiCl_2$	0·059
Cadmium, as $CdCl_2$	0·059	Potassium, as K_2SO_4	0·061
Calcium, as $CaCl_2$	0·062	Sodium, as Na_2SO_4	0·058
Cobalt, as $CoCl_2$	0·058	Strontium, as $SrCl_2$	0·058
Lead, as $Pb(C_2H_3O_2)_2$	0·061	Zinc, as $ZnCl_2$	0·060

Lead, barium and strontium were not precipitated as sulphates in these tests because the solutions were mixed rapidly as soon as the corresponding salt had been added (this was done to ensure that no lakes would be produced by these elements). In the proposed procedure, barium, lead and strontium were precipitated when the solution was diluted after

being heated to fumes with sulphuric acid. However, this is harmless, since, in the presence of these metals (and also of silica) the solutions are set aside for 1 hour after dilution to permit the precipitate to settle.

The effects of anions were investigated by placing 0·1-g amounts of different salts in 50-ml calibrated flasks containing 0·060 mg of tin and then developing the colour. (This amount of added salt is equivalent to 5 g in the original 250 ml.) The results are shown in Table III, from which it can be seen that nine anions did not interfere. Nitrite, sulphide,

TABLE III

RECOVERY OF TIN IN PRESENCE OF VARIOUS ANIONS

Anion present	Salt added*	Recovery of 0·06 mg of tin, mg
Acetate	$NaC_2H_3O_2$	0·062
Bromide	$NaBr$	0·058
Chlorate	$NaClO_3$	0·059
Chloride	$NaCl$	0·060
Nitrate	$NaNO_3$	0·058
Perchlorate	NH_4ClO_4	0·062
Phosphate	Na_2HPO_4	0·058
Sulphate	Na_2SO_4	0·060
Tartrate	$(NH_4)_2C_4H_4O_6$	0·063
Nitrite	$NaNO_2$	0·023
Sulphide	Na_2S	0·00
Sulphite	Na_2SO_3	0·00
Thiosulphate	$Na_2S_2O_3$	0·00†
Chromate	$Na_2Cr_2O_7$	0·036
Iodate	$NaIO_3$	0·018
Fluoride	KF	0·036
Oxalate	$Na_2C_2O_4$	0·034
Thiocyanate	$NaCNS$	0·030
Iodide	KI	0·071
Permanganate	$KMnO_4$	Manganese dioxide precipitated
Carbonate	Na_2CO_3	0·038
Persulphate	$(NH_4)_2S_2O_8$	0·074

* The amount of each salt added was 0·1 g, not including water of crystallisation.
† Visual estimation because some sulphur was precipitated.

sulphite and thiosulphate caused low results because of their reducing action, and chromate and iodate caused low results because of their oxidising action. Fluoride, oxalate and thiocyanate produced low results by forming complexes with the tin. Iodide interfered because iodine was liberated, and permanganate because manganese dioxide was precipitated. Carbonate caused low results, probably owing to neutralisation of the acid, and persulphate, for some unknown reason, led to high results. Interference from nitrite, sulphide, sulphite, thiosulphate, fluoride, oxalate, thiocyanate, carbonate and persulphate would be overcome by the heating to fumes in the recommended method (a platinum dish would have to be used with fluoride). The non-interference of chloride, perchlorate, and phosphate would seem to indicate that the corresponding acids (or mixtures of these with sulphuric acid) might be used for adjusting the acidity before taking the aliquot; this was not investigated.

The proposed procedure will probably be of great value for the colorimetric determination of other metals (especially aluminium, antimony, germanium, thorium and zirconium) as well as tin. It must be emphasised that the colours of the lakes mentioned in Table I were obtained with 1-mg amounts of the elements; for smaller amounts, the colours would probably resemble the brown and amber colours obtained with tin. The violet-coloured lakes were produced when there was a large excess of the metal over the dye; 1 mg of tin also produced a violet lake.

APPLICATION TO PROPELLANTS

For application to propellants, a 0·3-g sample was dissolved in a mixture of 10 ml each of nitric and sulphuric acids, and 1 ml of perchloric acid, and the solution was heated to fumes for 12 minutes. The perchloric acid destroyed the organic material. The solution was diluted to 250 ml, and a 5-ml aliquot was used for the proposed method. A typical propellant, containing 89·02 per cent. of nitrocellulose, 0·65 per cent. of diphenylamine, 0·94 per cent. of potassium sulphate, 6·71 per cent. of dinitrotoluene, 0·90 per cent. of moisture

and 0·95 per cent. of tin (as determined iodimetrically[1]), was analysed several times; the tin contents found, which show good accuracy and precision, were 0·98, 0·95, 1·00, 0·95, 0·95, 0·98 and 1·00 per cent. (average, 0·97 per cent.; standard deviation, 0·023 per cent.). Good recoveries (see Table IV) were obtained when standard tin solution A was added to 0·3-g portions of a propellant not containing tin and the samples were subjected to the proposed procedure. Results were satisfactory for prepared samples containing lead stearate, barium nitrate and strontium nitrate, and these recoveries are also shown in Table IV.

Table IV

Recovery of tin added to prepared samples

The M-9 propellant used contained 57·75 per cent. of nitrocellulose, 40·0 per cent. of nitroglycerine, 1·5 per cent. of potassium nitrate and 0·75 per cent. of diphenylamine

Sample		Tin found, mg
M-9 propellant (0·3 g)		0·00
M-9 propellant (0·3 g) *plus*	0·50 mg of tin	0·45
	1·00 mg of tin	1·00
	2·00 mg of tin	2·05
	3·00 mg of tin	3·05
	4·00 mg of tin	4·05
	5·00 mg of tin	4·90
M-9 propellant (0·3 g) *plus* tin (3·00 mg) *plus*	0·03 g of lead stearate	3·05
	0·015 g of barium nitrate	3·05
	0·015 g of strontium nitrate	2·95

Experiments were also carried out on a separation method whereby tin was first isolated as metastannic acid. This separation method offered no advantage over the direct method, but it did help to establish the validity of the direct method, as the same results were obtained. In this method, a 1·2-g sample was treated with 35 ml of nitric acid, the solution was evaporated to 10 ml, 50 ml of water were added, and boiling was continued for 5 minutes. The solution was then immediately filtered through a fine filter-paper, and the precipitate was washed with hot water. The precipitate and filter-paper were treated with a mixture of 10 ml of sulphuric acid, 15 ml of nitric acid and 3 ml of perchloric acid, and the solution was heated to dense fumes for 15 minutes and diluted to 1 litre after a further 30 ml of sulphuric acid had been added. A 5-ml aliquot of this solution was used for the determination.

The methods described above were not applicable to the determination of tin dioxide, which was insoluble in fuming sulphuric acid. In an attempt to develop a method applicable to metallic tin and to tin dioxide, much work was done on a fusion procedure whereby a 0·3-g sample was treated with 5 ml of nitric acid in a platinum crucible, the solution was evaporated to dryness, and the residue ignited for 3 minutes. The residue was then fused with 8 g of potassium hydrogen sulphate, and 10 ml of sulphuric acid were added 25 seconds after turning out the flame (in this way no salts were precipitated). The solution was then diluted to 250 ml, and a 5-ml aliquot was used. Although the method seemed attractive in principle, the precision was poor ($\pm$0·2 per cent.), probably because of the potassium hydrogen sulphate and the platinum picked up during the fusion. The method may be useful for determining tin dioxide in propellants when only a small amount of sample is available (the gravimetric method is not satisfactory for small samples). In applying the fusion method, it is essential that the platinum crucible be free from contamination by iron.

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The Determination of Dithionate, Sulphite and Sulphate in Manganese Leach Liquors

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A rapid method is described for determining dithionate, sulphite-S dioxide and sulphate present together in manganese leach liquors; it consists in titrimetric determinations on three separate aliquots of sample. (*a*) Dithionate is decomposed by treatment with a relatively large amount of hydrochloric acid, and the solution is kept boiling under an atmosphere of carbon dioxide in a standard apparatus. The sulphur dioxide evolved is expelled by a continuous stream of carbon dioxide into a cooled excess of standard iodine solution, and the unconsumed iodine is titrated; sulphite-S dioxide is expelled before the determination or its independently determined content is deducted from the result. (*b*) Sulphite-S dioxide is determined iodimetrically after chelation of interfering cations with disodium ethylenediaminetetra-acetate solution. (*c*) Sulphate is determined, after removal of interfering cations by ion exchange, by precipitation with an excess of standard barium chloride solution at room temperature and then titration of the unconsumed barium with disodium ethylenediaminetetra-acetate solution.

The method has been applied to leach liquors containing, per litre, 85 to 90 g of manganese, 0·001 to 2·5 g of iron, 0·05 to 1·5 g of copper, 3 to 70 g of dithionate, up to 11 g of sulphur dioxide (including sulphite as sulphur dioxide) and 110 to 240 g of sulphate.

DURING work on the preparation of high-grade manganese concentrates[1] via sulphur dioxide wet leaching of pyrolusitic manganese ore, it was necessary to determine dithionate, sulphite and sulphate ions in presence of each other. These ions, together with manganese, were the main consistuents of the leach liquors, and their determination by existing methods was not feasible; in particular, the analysis of liquors having extreme ratios of concentrations was an outstanding problem. The main problems are briefly discussed below.

Dithionate in acid medium and at elevated temperature decomposes[2 to 9] into sulphate and sulphur dioxide in accordance with the equation—

$$S_2O_6^{2-} = SO_4^{2-} + SO_2 \quad .. \quad .. \quad .. \quad .. \quad (1)$$

and this decomposition has formed the basis for its determination.[4,10,11]

Dithionate can also be quantitatively oxidised to sulphate according to the equation—

$$S_2O_6^{2-} + O_2 = 2SO_4^{2-} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

and several workers[12 to 22] have determined dithionate in this way, both dry and wet methods of oxidation being used. However, in presence of sulphate, the determination of dithionate via the determination of sulphate formed in accordance with equations (1) and (2) is not feasible unless the original sulphate content is known. Further, when a wet-oxidation procedure is used and the excess of oxidising agent is determined volumetrically, several metal ions, chloride and sulphite interfere; both dry and wet methods of oxidation are tedious and lengthy. Moreover, in presence of dithionate, the gravimetric determination of sulphate (as barium sulphate) is not possible, owing to partial decomposition of dithionate under the conditions necessary for determining sulphate. In some of the procedures referred to above sulphate was determined gravimetrically at 60° C in order to prevent decomposition of dithionate; however, even at 60° C and low acidity, dithionate was partly decomposed.

The method finally adopted for determining dithionate was based on iodimetric determination of the sulphur dioxide formed in equation (1). Sulphur dioxide formed from sulphites originally present in the liquor was expelled beforehand in the cold or an independently determined result for it was deducted from the figure for total sulphur dioxide. Hydrochloric acid was used for the decomposition, so that the sulphate content of the residue after decomposition consisted of sulphate originally present and sulphate resulting from equation (1). The sulphate originally present was independently determined by the procedure described on p. 845, and the sulphate produced from the dithionate was calculated by difference.

Sulphite-S dioxide in the liquor was determined iodimetrically after interfering cations had been removed by formation of complexes with the disodium salt of ethylenediaminetetra-acetic acid (EDTA) in acidic medium.

Sulphate in the leach liquors was determined by running an aliquot of sample (free from metals) into an excess of standard barium chloride solution at room temperature, the unconsumed barium being titrated with standard EDTA solution.

Determination of dithionate

Reagents—

Iodine, 0·1 N.
Sodium thiosulphate, 0·1 N.
Starch solution, 1 *per cent. w/v.*
Hydrochloric acid, concentrated—Analytical-reagent grade.
Potassium iodide—Analytical-reagent grade.

Procedure—

Introduce 20 to 25 ml of 0·1 N iodine into flask A (see Fig. 1), add water until the flask is two-thirds full, and cool in an ice - water bath. In flask B place 0·2 to 0·3 g of potassium iodide dissolved in about 200 ml of water, and add starch solution as indicator. Place the aliquot of sample (adjusted, if necessary, to the convenient volume of about 20 ml) in flask C, pass carbon dioxide[23 to 26] from a cylinder in at D, and add 10 ml of concentrated hydrochloric acid via E. After 2 or 3 minutes, heat flask C until its contents distil, with a continuous stream of carbon dioxide passing through at about 2 ml per second; sudden boiling should be avoided. (The distillation should take 5 to 10 minutes and be continued almost to dryness.) Allow carbon dioxide to pass for several minutes more, and disconnect the apparatus (with carbon dioxide passing), beginning at E and continuing via F and G. Rinse those parts that have been in contact with the iodine solution, and also rinse the inner tube of the condenser. Titrate the excess of iodine with 0·1 N sodium thiosulphate, and also titrate any iodine that has escaped into flask B (as evidenced by the formation of a blue colour).

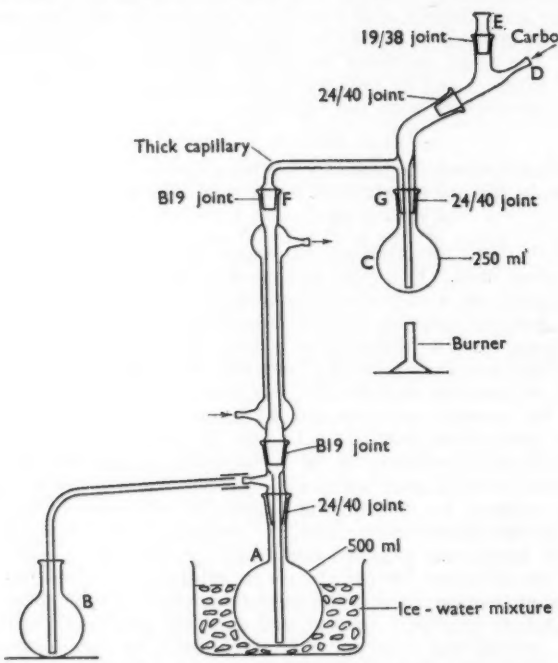

Fig. 1. Apparatus used for determining dithionate

Determination of sulphite-S dioxide

The procedure used for this determination has been described by Mashall[27] and, as the oxidation of sulphite to sulphate is catalysed by manganese sulphate,[28,29] should be carried out without undue delay.

Procedure—

Prepare a solution containing 100 ml of water, 3 ml of concentrated hydrochloric acid and 10 to 15 ml (depending on the amount of interfering ions present) of 0·2 M EDTA solution (see Note). To this solution add an excess of 0·1 N iodine, and slowly add the aliquot of sample from a pipette; shake the flask during the addition. Titrate the excess of iodine with 0·1 N sodium thiosulphate.

Note—The minimum acidity below which ferrous iron interferes is 0·3 M in hydrochloric acid; at this acidity, interference from ferric and cupric ions can be overcome by treatment with an amount of EDTA solution at least 50 per cent. in excess of that stoicheiometrically necessary.

Determination of sulphate

Reagents—

EDTA solution, 0·01 M—Dissolve 14·6 g of disodium ethylenediaminetetra-acetate, 2 g of magnesium chloride hexahydrate and 1·1 g of calcium chloride (calculated as the anhydrous salt) in water, dilute to 2 litres, and standardise against 0·2 N barium chloride.

Eriochrome black T indicator mixture—Triturate a mixture of 1 part of the indicator and 99 parts of sodium chloride.

Barium chloride, 0·2 N.

Buffer solution—Dissolve 60 g of ammonium chloride in about 200 ml of water, add 570 ml of ammonia solution, sp.gr. 0·880, and dilute to 1 litre.

Standardisation of EDTA solution—

To 2 ml of the 0·2 N barium chloride add 50 ml of freshly boiled distilled water, 5 ml of the buffer solution and about 10 mg of the indicator mixture, and titrate with the EDTA solution to the blue end-point.

1 ml of 0·01 M EDTA solution (factor = 1·000) ≡ 0·9606 mg of sulphate.

$$\text{Factor for EDTA solution} = \frac{\text{0·2 N barium chloride present, ml} \times 10}{\text{Titre of EDT solution, ml}}.$$

Procedure*—

By pipette, transfer an aliquot of the sample containing 40 to 90 mg of sulphate to a 35-cm × 1·5-cm (radius) column of a strongly acid cation-exchange resin in the hydrogen form. Add 30 to 40 ml of freshly boiled water, and allow it to pass through at the rate of 40 to 50 drops per minute. Collect the effluent in a 250-ml calibrated flask containing 10 ml of 0·2 N barium chloride, and wash the column until the effluent is neutral. Dilute the combined effluent and washings to the mark, and set aside for not less than 30 minutes. By pipette, place 100 ml of the clear supernatant liquid in an Erlenmeyer flask, add 5 ml of the buffer solution and then 10 mg of indicator mixture, and immediately titrate with 0·01 M EDTA solution to the blue end-point. Calculate the sulphate content of the sample from the equation—

$$\text{Sulphate content, g per litre} = \frac{10A - (B \times F \times 2{\cdot}5)}{V}$$

in which A is the amount of sulphate (in milligrams) equivalent to 1 ml of the 0·2 N barium chloride, B is the titre of EDTA solution (in millilitres), F is the amount of sulphate (in milligrams) equivalent to 1 ml of 0·01 M EDTA solution and V is the volume of the sample aliquot (in millilitres).

Note that interference from sulphite, when present in relatively large amount, can be avoided by destroying the sulphite at room temperature in a slightly acid medium before the sulphate is determined. Sulphite is slowly oxidised to sulphate under the conditions of the determination described above.

* See also paper by Munger, Nippler and Ingols.[30]

Results

The methods for dithionate and sulphate were tested by applying them to prepared stock solutions; sulphite-S dioxide is unstable (oxidation to sulphate),[31] so that its evaluation in such solutions is of no value.

Test of method for determining sulphate—

A stock solution of manganese sulphate (free from sulphite) was prepared and analysed. The manganese content, determined potentiometrically,[32] was 0·080 equivalent per litre; the sulphate content, determined gravimetrically (single precipitation) and by the proposed method, was also found to be 0·080 equivalent per litre.

Test of method for determining dithionate—

A stock solution of manganese dithionate was prepared by leaching manganese dioxide as described by Fernelius[33]; the manganese dioxide (obtained from May and Baker Ltd.) had a purity of 94·5 per cent. and a total manganese content of 60·4 per cent. The sulphate in the leach liquor was precipitated by adding a solution of analytical-reagent grade hydrated barium hydroxide, and an accidental excess of barium was then removed with sulphuric acid, so that the resulting dithionate solution was acid. This solution, which was free of reducing agents, was analysed potentiometrically for manganese and alkalimetrically (after the addition of ammonium chloride and with methyl orange as indicator) for hydrogen ion. Its dithionate content was determined by the five procedures listed below.

(*i*) By the proposed method.

(*ii*) The solution was allowed to pass through a cation-exchange resin, and dithionate in the effluent was determined by the proposed method.

(*iii*) The residue from (*i*) was dissolved in water, and the sulphate content of this solution was determined by the proposed method. The dithionate content was then calculated from equation (1), p. 843.

(*iv*) As in (*iii*), but the sulphate content was determined gravimetrically (single precipitation).

(*v*) An aliquot of the solution (containing about 8×10^{-4} equivalent of dithionate) was treated with 0·2 g of potassium iodate and 15 ml of concentrated hydrochloric acid. The solution was carefully evaporated to about 2 ml, its sulphate content was determined gravimetrically (single precipitation), and the dithionate content was calculated from equation (2), p. 843.

The results are shown in Table I.

Table I

Composition of stock dithionate solution

Cations		Dithionate	
Present	Concentration found, equivalents per litre	Procedure No.*	Concentration found, equivalents per litre
		(*i*)	0·875
Manganese	0·713	(*ii*)	0·870
Hydrogen	0·163	(*iii*)	0·874
Total	0·876	(*iv*)	0·876
		(*v*)	0·879

* See text.

Determination of dithionate and sulphate present together—

For each of these tests, 1 ml of the stock dithionate solution was diluted to 10 ml with water and mixed with 10 ml of the stock manganese sulphate solution. Dithionate was determined in the mixed solution by four of the five procedures outlined above; in procedure (*iii*), the dithionate content was calculated from the sulphate content found by the proposed method. The dithionate contents (calculated for the original stock solution) found by procedures (*i*), (*iii*), (*iv*) *and* (*v*) were 0·870, 0·875, 0·882 and 0·883 equivalent per litre, respectively. The sulphate content of the stock manganese sulphate solution, calculated

from the result obtained when the proposed method for sulphate was applied to the mixed solution, was found to be 0·0796 equivalent per litre.

In these determinations the ratio of dithionate to sulphate in the mixed solutions was about 1 to 1 in equivalents, and a second series of determinations was carried out with this ratio fixed at about 0·1 to 1. The stock dithionate solution was diluted to a concentration of 0·0088 equivalent per litre, and each of four 10-ml aliquots of this solution was mixed with 10 ml of the stock manganese sulphate solution. The dithionate contents of the mixed samples were then determined by the proposed procedure, with certain modifications. These modifications were (*a*) the use of 0·01 N standard solutions, (*b*) the addition of sodium hydrogen carbonate when the end-point of the titration had almost been reached,[22,34] so forming a layer of carbon dioxide over the solution, and (*c*) the use of between 2·5 and 5 ml of 0·1 N hydrochloric acid for decomposing the dithionate. The results, calculated for the stock solution, were 0·88, 0·87, 0·88 and 0·87 equivalent of dithionate per litre.

Analysis of manganese leach liquors

The determination of dithionate, sulphite-S dioxide (expressed as sulphur dioxide) and sulphate in the presence of each other was carried out on a series of manganese leach liquors; in each of the experiments described below, a different liquor was used.

Analysis of a liquor low in dithionate and relatively high in sulphite-S dioxide—

The sulphite-S dioxide and dithionate were determined by the proposed procedures in two separate aliquots of the same leach liquor. Sulphur dioxide was not expelled before dithionate was determined, and the mean result for sulphur dioxide was used to calculate the dithionate content according to equation (1); the results were—

Sample No.	1	2
Sulphite-S dioxide (as SO_2), g per litre	6·02	6·05
Dithionate, g per litre	2·93	2·93

Effect of instability of sulphite-S dioxide—

The presence of dissolved oxygen in the liquor, as produced by dilution, causes a decrease in sulphur dioxide content; this decrease becomes more pronounced with time, as shown by the results in Table II.

Table II

Sulphite-S dioxide, dithionate and sulphate contents found at various times

Sample	Sulphite-S dioxide content (as SO_2), g per litre	Dithionate content, g per litre	Sulphate content, g per litre
Leach liquor No. 1	14·41	53·3	122
Leach liquor No. 1 immediately after dilution	13·45	52·8	123
Leach liquor No. 2	12·50	60·0	115·7
Leach liquor No. 2 after a few weeks	10·90	60·4	118·1

Table III

Composition of a leach liquor

The liquor contained 5·21 equivalents of manganese per litre; its total-cation content (as hydrogen ion) found by cation exchange was 5·28 equivalents per litre

Sample No.	Sulphite-S dioxide content (as SO_2), g per litre	Dithionate content, g per litre	Sulphate content, g per litre
1	1·77	130·8	173·4
2	1·77	129·5	173·4
			174·0*
Mean, equivalents per litre	0·05	1·625	3·61
Total anions, equivalents per litre		5·285	

* Result found gravimetrically on residue from decomposition of dithionate.

CATION - ANION BALANCE—

The sulphur-containing constituents were determined by the proposed procedures on two aliquots of a liquor, and a control determination of sulphate was carried out gravimetrically on the residue from the decomposition of dithionate in one of the aliquots. Comparison of this result with that for directly determined sulphate (see Table III) provided confirmation of the figures for dithionate and sulphate.

COMPLETE ANALYSIS OF LEACH LIQUOR—

The sulphur-containing constituents of a leach liquor were determined by the proposed procedures, sulphite-S dioxide being first expelled; further, the dithionate in a separate aliquot was oxidised by potassium iodate, and sulphate was determined gravimetrically in this solution. The dithionate content was then calculated from this result after correction for sulphate originally present in the liquor. The results are shown in Table IV.

TABLE IV

RESULTS FOR COMPLETE ANALYSIS OF A LEACH LIQUOR

Cation	Concentration of cation found per litre		Anion	Concentration of anion found per litre	
	g	equivalents		g	equivalents
Manganese	90·9	3·310	Chloride	1·55	0·043
Copper	0·056	—	Sulphate	139	2·896
Iron	0·002	—	Dithionate	43·3	0·541,
Calcium	0·69	0·034		—	0·541*
Magnesium	0·26	0·022	Total anions	..	3·480
Sodium	1·06	0·046			
Potassium	0·16	0·004			
Aluminium	Nil	—			
Total cations	..	3·416†			

* Calculated from determination of total sulphate after oxidation.
† Total-cation content found by ion exchange = 3·47 equivalents per litre.

DISCUSSION OF RESULTS

The accuracies of the three methods have been tested and confirmed by using prepared solutions and manganese leach liquors. Accuracy was satisfactory for solutions containing proportions of the ions corresponding to the extreme concentrations found in leach liquors.

The reproducibilities of the methods were also tested and found to agree well with those stated in the literature for the iodimetric titration of dithionate and sulphite-S dioxide and for the volumetric determination of sulphate. These results have not been included, as the main aim was to describe the application of three modified and adapted procedures to the analysis of leach liquors. (The instability of sulphite-S dioxide in leach liquors should be borne in mind when testing reproducibility.)

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The Determination of Propane-1,3-diol in Glycerol by Gas Chromatography with a Flame-ionisation Detector

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A simple method is described for determining propane-1,3-diol in glycerol by gas chromatography. A flame-ionisation detector is employed, and this permits the method to be used for analysing dilute aqueous solutions, as the detector is insensitive to water.

Clifford has described a method[1] for determining propane-1,3-diol, *i.e.*, trimethyleneglycol, in glycerol by gas chromatography; he used a column containing 25 per cent. of Reoplex 400, as liquid phase, dispersed on Celite (60 to 85 mesh). The gas-chromatographic method is direct and overcomes the difficulties present in older methods,[2,3] which are based on distillation and then determination of specific gravity. Such methods may include impurities other than trimethyleneglycol, thereby leading to erroneous results.

We confirmed Clifford's results with use of an argon-ionisation detector, but considered that there were a number of disadvantages in the use of this detector, which imposed limitations on the type of glycerol sample that could be analysed. For example, as Clifford pointed out, an argon-ionisation detector is to some degree sensitive to water, and, although the effect is not sufficiently great to cause interference with the glycerol samples normally met in practice, it would not be possible directly to analyse dilute aqueous solutions. Attention was therefore turned to the possible use of a flame-ionisation detector,[4] which is almost completely insensitive to water and would therefore considerably increase the scope of the method. Further, a glass flame-ionisation detector can be readily constructed in the laboratory. The gas-chromatographic determination of trimethyleneglycol in glycerol has been examined under conditions similar to those employed by Clifford, but they were made to conform with those normally used with flame-ionisation detectors, *e.g.*, the amplifier used in conjunction with the argon-ionisation detector was slightly modified for use with the flame detector. It was found that diethyleneglycol could be used as internal standard, and comparison of the heights of the peaks for this glycol and trimethyleneglycol readily led to the determination of the amount of the latter in the sample. In contrast, Clifford's method,

with an argon detector, involves comparison of the small area under the peak for trimethyleneglycol (which is not so sharp as when a flame-ionisation detector is used) with the large area under the peak for glycerol.

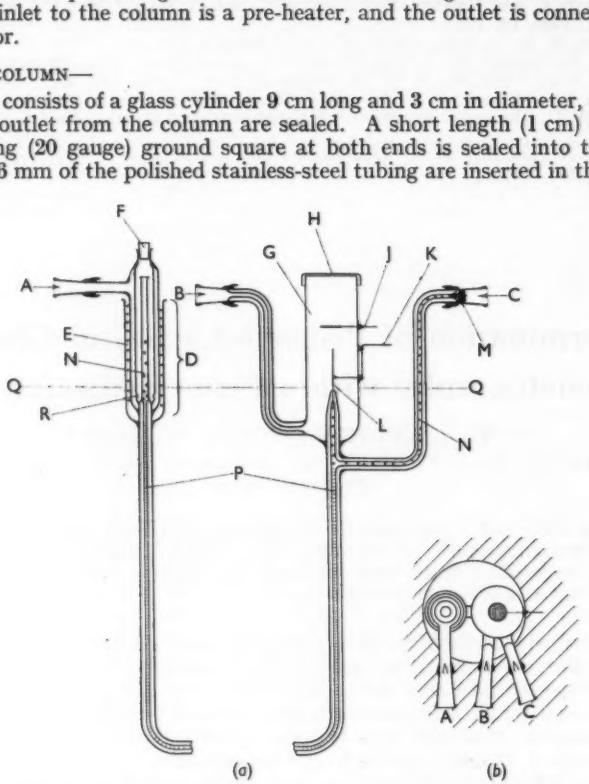
Aqueous solutions containing 10 per cent. of the glycerol sample were used, but there is no reason to suppose that solutions as dilute as 1 per cent. with respect to glycerol may not be analysed. With samples of glycerol containing from about 0·2 to 2·5 per cent. of trimethyleneglycol, an accuracy to better than ± 10 per cent. is attainable.

Description of apparatus

The apparatus comprises a glass column of over-all length 28 cm and 2 mm internal diameter; at the inlet to the column is a pre-heater, and the outlet is connected to a flame-ionisation detector.

Detector and column—

The detector consists of a glass cylinder 9 cm long and 3 cm in diameter, and into its base the air inlet and outlet from the column are sealed. A short length (1 cm) of stainless-steel hypodermic tubing (20 gauge) ground square at both ends is sealed into the outlet of the column. (About 6 mm of the polished stainless-steel tubing are inserted in the glass outlet of



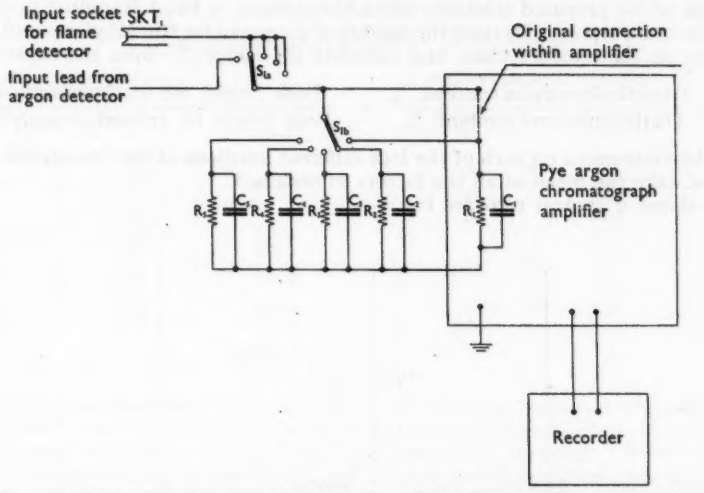
A = Nitrogen inlet (C7 ground-glass socket)
B = Air inlet (C7 ground-glass socket)
C = Hydrogen inlet (C7 ground-glass socket)
D = Preheater
E = Nichrome-wire heater
F = Silcoset serum cap
G = Detector (cylinder 9 cm long, 3 cm diameter)
H = Glass cover
= Platinum gauze positive electrode (1 cm diameter and situated 1 cm from top of K)
K = Platinum connection to hypodermic tubing (negative electrode)
L = Hypodermic tubing (20 gauge, 1 cm long)
M = Glass-fibre plug
N = Ballotini glass spheres
P = Reoplex 400 supported on Embacel
Q = Top of oven
R = Nitrogen inlet to inner wall of preheater

Fig. 1. (*a*) Column incorporating flame-ionisation detector; (*b*) plan view showing arrangement of side-arms

the column, which is then heated sufficiently just to allow the glass to shrink on to a few millimetres of the metal surface, so that about 3 mm of the steel tubing project into the column; the metal-to-glass seal so constructed is gas-tight, provided that it is not subjected to shock.) A piece of 22-gauge platinum wire welded on to a short length of 18-gauge wire is attached to the hypodermic tubing and forms the negative electrode; the 18-gauge wire is sealed into the wall of the glass cylinder. The positive electrode consists of a piece of 18-gauge wire sealed into the cylinder and terminating inside in a loop, to which is welded a small disc of fine platinum gauze. The cylinder is closed with a loose-fitting glass cover. The pre-heater attached to the inlet of the column consists of a double-jacketed unit heated by a simple Nichrome-wire-wound heater. Details of the various dimensions are shown in Fig. 1.

The column is filled with 25 per cent. of Reoplex 400 (obtained from Geigy Co. Ltd.) as liquid phase dispersed on 60- to 100-mesh Embacel kieselguhr (obtained from May and Baker Ltd.) to a level just below the pre-heater and then with Ballotini glass spheres, or some similar inert material, to the level of the centre of the pre-heater. The sample inlet of the pre-heater is closed by means of a silicone-rubber serum cap (Silcoset 102; obtainable from Imperial Chemical Industries Ltd.). Fig. 1 (*a*) shows an expanded view of the column, and the true positions of the nitrogen, air and hydrogen inlets are shown in the plan view, Fig. 1 (*b*). The column and detector are housed in an air-heated oven (0° to 250° C) of a type similar to those obtainable from Griffin and George Ltd.

The flows of hydrogen and nitrogen are controlled by means of Edwards' VPC 1 diaphragm-operated valves, and the flow of air by means of a Foxboro reducing valve operating in the range 0 to 30 lb per sq. inch. The supplies of hydrogen and air are filtered through charcoal contained in small tubes and supported on sintered-glass discs (porosity No. 1).



R_1 = Original resistor for argon detector
R_2 = 10-megohm Welwyn C.23 resistor
R_3 = 100-megohm Welwyn H.11 resistor
R_4 = 1000-megohm Welwyn H.12 resistor
R_5 = 10,000-megohm Welwyn H.12 resistor

C_1 = Original capacitor for argon detector
C_2, C_3, C_4 = 2000-pF T.C.C. Plastapack capacitor
C_5 = 200-pF T.C.C. Plastapack capacitor
SKT_1 = Belling-Lee co-axial socket
S_1 = Ceramic 2-pole 5-way wafer switch

Fig. 2. Modifications to Pye amplifier

AMPLIFIER FOR OPERATION OF DETECTOR—

A Pye amplifier used with the argon-ionisation detector was modified so that it could also be used with the flame-ionisation detector. The modifications were made to the input circuit and are shown in Fig. 2. Thus, a ceramic switch was installed so that the amplifier could be connected either to the input of the argon detector or to an alternative coaxial socket on the front panel, leading to the flame detector. The switch has one "argon-detector" position, in which the original 350-megohm input resistor remained in circuit, and in the

modification (see Fig. 2) are four "flame-detector" positions to provide a choice of input resistor, *viz.*, 10, 100, 1000 or 10,000 megohms. In the last (most sensitive) position, the capacitor shunting the input resistor in the original circuit was decreased from 2000 to 200 picofarads in order to decrease the time constant and avoid sluggishness in the response of the amplifier.

Method

Column conditions—

Column temperature—150° C.
Pre-heater temperature—175° C.
Carrier-gas (nitrogen) flow—10 ml per minute.
Hydrogen flow to flame—15 ml per minute.
Air-feed flow—500 ml per minute.
Electrode potential—126 volts.

Calculation of factors—

Prepare a 10 per cent. w/v solution of pure glycerol in water, and use this solution to prepare standard solutions containing 2 per cent. w/v of trimethyleneglycol and diethyleneglycol. From these solutions prepare four mixtures containing 10 per cent. w/v of glycerol, 0·2 per cent. of diethyleneglycol and different concentrations of trimethyleneglycol between 0·02 and 0·2 per cent.; these cover the range 0·2 to 2 per cent. of trimethyleneglycol in glycerol.

Before operating the column at 150° C, remove volatile material by heating to 200° C for 3 to 4 hours. With the apparatus working under the conditions listed above, inject 2-μl samples of the prepared solutions on to the column; a 10-μl Hamilton syringe pipette is suitable for this purpose. Measure the heights of the peaks for trimethylene- and diethyleneglycol shown on the recorder trace, and calculate the factor (F) from the expression—

$$F = \frac{\text{Trimethyleneglycol content, \%}}{\text{Diethyleneglycol content, \%}} \times \frac{\text{Peak height for diethyleneglycol}}{\text{Peak height for trimethyleneglycol}}$$

Make two determinations on each of the four different solutions of the two glycols in aqueous glycerol, and take the mean of all the factors so obtained.

Fig. 3 shows a typical recorder trace.

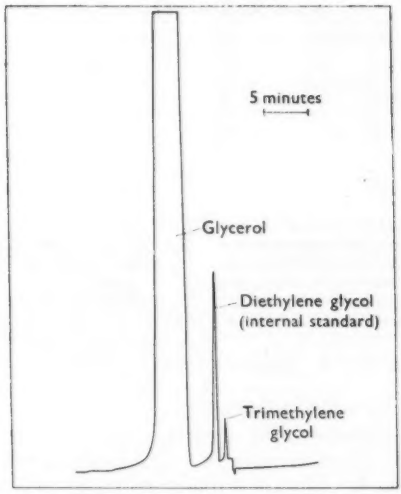


Fig. 3. Chromatogram of sample of glycerol containing 0·26 per cent. of trimethyleneglycol

PROCEDURE FOR ANALYSING GLYCEROL—

To 10 g of the glycerol sample add 10 ml of an aqueous 2 per cent. w/v solution of diethyleneglycol, and dilute to 100 ml with water. By means of a Hamilton syringe pipette, transfer 2 μl of this solution to the column, and record a chromatogram. Measure the heights of the peaks for trimethylene- and diethyleneglycol, and use the factor already found to calculate the trimethyleneglycol content of the sample from the expression—

$$\text{Trimethyleneglycol content, \%} = \frac{\text{F (average)} \times 2{\cdot}0 \times \text{Peak height for trimethyleneglycol}}{\text{Peak height for diethyleneglycol}}$$

RESULTS

RETENTION TIMES—

With a Reoplex - Embacel column, retention times under the conditions described above were 0·7 minute for trimethyleneglycol and 1·6 minutes for the diethyleneglycol used as standard; a determination is complete in 14 minutes, which is the time taken for elution of the glycerol.

DETERMINATION OF TRIMETHYLENEGLYCOL IN GLYCEROL—

Results found for various samples of glycerol containing from 0·26 to 2·51 per cent. of trimethyleneglycol are shown in Table I and are considered to be satisfactory; the single sets of determinations were made on different days.

TABLE I

TRIMETHYLENEGLYCOL FOUND IN GLYCEROL

Trimethyleneglycol content— present, %	Trimethyleneglycol content— found, %	Factor used	Trimethyleneglycol content— present, %	Trimethyleneglycol content— found, %	Factor used
0·26	0·28	0·685	1·22	1·09	0·685
	0·26			1·19	
	0·28		1·22	1·21	0·684
	0·26		1·22	1·26	0·657
0·26	0·30	0·684	2·51	2·69	0·685
0·26	0·29	0·657		2·85	
0·67	0·68	0·685		2·57	
	0·71			2·58	
0·67	0·70	0·684		2·56	
0·67	0·75	0·657	2·51	2·40	0·657

CONCLUSIONS

A sensitive gas-chromatographic method for determining trimethyleneglycol in glycerol has been devised. An important feature of the method is the use of dilute aqueous solutions, since the detector employed is almost completely insensitive to water. More recent experiments have indicated that the sensitivity and accuracy of the method can be still further improved by using a flame-ionisation detector constructed in metal.

We thank Mr. W. Young of this Department for the electrical modification of the amplifier.

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Notes

DETERMINATION OF ALKALI POLYSULPHIDES

SULPHUR reacts with alkali hydroxide to produce sulphide, polysulphide and thiosulphate[1] in accordance with the equation—

$$(4 + 2x)\,S + 6OH^- = 2(S.S_x)^{2-} + S_2O_3^{2-} + 3H_2O$$

The resulting solution can be analysed by several methods,[2,3,4,5] but the determination of alkali polysulphides has remained a problem. Kurtenacker developed a method for determining sulphide in the presence of thiosulphate and sulphite[6]; sulphide was separated from thiosulphate and sulphite with a suspension of basic zinc carbonate, after which sulphite was converted into formaldehyde - bisulphite, and thiosulphate was determined iodimetrically. The sum of the sulphide, thiosulphate and sulphite was determined iodimetrically on a separate sample. Thiosulphate has also been determined in presence of sulphide and sulphite.[7,8] In titrations with iodine, thiosulphate is oxidised to tetrathionate, which is allowed to react with cyanide, so producing thiosulphate according to the equation—

$$S_4O_6^{2-} + 3CN^- + H_2O = S_2O_3^{2-} + SO_4^{2-} + SCN^- + 2HCN$$

and this thiosulphate is again titrated with iodine. (There are no corresponding reactions between cyanide and the oxidation products of sulphide or sulphite.) All these methods can be used for determining alkali polysulphides. In the analysis of lime sulphur solution, an ammoniacal solution of zinc chloride has been used for the precipitation of polysulphide- and sulphide-S.[9]

A method developed for the titrimetric determination of alkali polysulphides is described in this Note. Sulphide and polysulphide are separated from thiosulphate by treatment with an ammoniacal solution of zinc sulphate, and polysulphide is determined in accordance with the equations—

$$Na_2(S.S_x) + Zn(NH_3)_6(OH)_2 = Zn(NH_3)_6(S.S_x) + 2NaOH$$

$$Zn(NH_3)_6(S.S_x) + 8HCl = ZnCl_2 + H_2S + xS + 6NH_4Cl$$

$$S + 2KOH + 3H_2O_2 = K_2SO_4 + 4H_2O$$

(A rapid and accurate method[10] developed for determining elemental sulphur has been based on the last of these equations.)

The thiosulphate is determined iodimetrically in the filtrate from the precipitation of zinc sulphide, and sulphide is determined indirectly by iodimetric titration of the alkali polysulphide solution. The total sulphur is determined in a separate sample by oxidation with bromine water, precipitation by barium ions[11] of the sulphate formed and titration of the excess of barium ions with EDTA solution, a mixture of Eriochrome black T and the sodium salt of rhodizonic acid being used as indicator.[12]

METHOD

REAGENTS—

Zinc sulphate, 0·4 N.

Ammonia solution, diluted (1 + 1)—Analytical-reagent grade.

Ethanolic potassium hydroxide solution—Dissolve 28 g of analytical-reagent grade potassium hydroxide in 1 litre of 96 per cent. ethanol; warm to assist solution.

Hydrogen peroxide solution, 30 *per cent. v/v*—Analytical-reagent grade.

Hydrochloric acid, N.

Sodium hydroxide, N.

Methyl red indicator solution, 0·2 *per cent. w/v.*

Iodine, 0·1 N.

Methyl orange indicator solution, 0·1 *per cent. w/v.*

Starch indicator solution, 0·5 *per cent. w/v.*

Sodium thiosulphate, 0·1 N.

Phenol solution, 5 *per cent. w/v.*

EDTA solution, 0·05 M.

Barium chloride, 0·1 M.

Buffer solution—Dissolve 8·25 g of ammonium chloride in water, add 113 ml of ammonia solution, sp.gr. 0·880, and dilute to 1 litre.

Eriochrome black T indicator solution—Dissolve 1 g of the indicator in 100 ml of triethanolamine.

Rhodizonic acid indicator solution, 0·4 per cent. w/v—Freshly prepare this solution from the sodium salt of the acid.

PROCEDURE FOR DETERMINING POLYSULPHIDE-S—

Add the diluted ammonia solution to 20 ml of 0·4 N zinc sulphate until the precipitate first formed re-dissolves, and then add the sample containing approximately 0·12 g of polysulphide-S. After 20 minutes, separate the precipitated zinc sulphide by filtration, and wash it with 100 ml of water to remove thiosulphate. Boil the washed precipitate and filter-paper for 30 minutes with a mixture of 10 ml of N hydrochloric acid and 25 ml of water, add 2 drops of methyl red indicator solution, and then, from a burette, add N sodium hydroxide until the pink colour disappears. Filter the solution (containing the filter-paper pulp) through a small filter-paper, thoroughly wash, and transfer precipitate and filter-paper to a beaker containing 50 ml of ethanolic potassium hydroxide solution. Heat on a steam-bath until the solution has almost evaporated (about 30 minutes). Cool, add 50 ml of water and 3 ml of the 30 per cent. solution of hydrogen peroxide, and, after 10 minutes, titrate with N hydrochloric acid; use methyl red as indicator. Carry out a blank determination on the potassium hydroxide solution, including filter-papers and filter-paper pulp.

1 ml of N hydrochloric acid ≡ 0·016033 g of polysulphide-S.

PROCEDURE FOR DETERMINING THIOSULPHATE-S—

Exactly neutralise the combined filtrates and washings from the precipitated zinc sulphide and polysulphide with N hydrochloric acid; use methyl orange as indicator. To this neutral solution add a few drops of starch indicator solution, and titrate with 0·1 N iodine.

1 ml of 0·1 N iodine ≡ 0·006413 g of thiosulphate-S.

PROCEDURE FOR DETERMINING SULPHIDE-S—

To a mixture of 20 ml of 0·1 N iodine and 10 ml of N hydrochloric acid add a portion of sample containing approximately 0·015 g of sulphide-S, and, after 10 minutes, titrate the solution with 0·1 N sodium thiosulphate; use starch as indicator.

In this reaction polysulphide-S is precipitated, as elemental sulphur, without consumption of iodine, but sulphide-S is oxidised by the iodine to elemental sulphur and thiosulphate to tetrathionate; the amount of iodine consumed is therefore a measure of the sulphide- and thiosulphate-S present. Subtract the thiosulphate-S found previously, and report the difference as sulphide-S.

1 ml of 0·1 N iodine ≡ 0·0016033 g of sulphide-S.

PROCEDURE FOR DETERMINING TOTAL SULPHUR—

To 10 ml of water add a weighed sample of alkali polysulphide containing 0·025 to 0·03 g of sulphur. Run into the solution 50 ml of bromine water, boil for 5 minutes until all the sulphur has dissolved, and add phenol solution until the yellow colour has practically disappeared (3 to 5 drops of the phenol solution will be necessary). Cool, add 25 ml of 0·1 M barium chloride, 10 ml of buffer solution, 3 drops of Eriochrome black T solution and 6 drops of rhodizonic acid solution,

TABLE I

RESULTS FOUND FOR ALKALI POLYSULPHIDE SOLUTIONS

Sulphide-S found by—		Thiosulphate-S found by—		Poly-sulphide-S found by proposed method,	Poly-sulphide-S calculated from total sulphur,	Total sulphur found by proposed method,
proposed method,	Kurtenacker's method,[6]	proposed method,	Kurtenacker's method,[7]			
g	g	g	g	g	g	g
Solution containing 30 g of sulphur—						
6·89	7·02	7·32	7·01	14·91	15·61	29·82
6·76	7·04	7·31	7·03	15·20	15·79	29·86
Solution containing 40 g of sulphur—						
8·81	9·31	9·10	9·22	22·02	22·07	39·98
8·79	9·32	9·08	9·42	21·64	21·95	39·82
Solution containing 45 g of sulphur—						
9·70	9·92	9·67	9·98	25·74	26·09	45·46
9·76	9·93	9·68	10·13	25·76	25·86	45·30

and titrate with 0·05 M EDTA solution until the colour changes from bluish red to yellowish green. The difference between the titre for 25 ml of 0·1 M barium chloride and that for the sample solution is equivalent to the total sulphur.

1 ml of 0·05 M EDTA solution ≡ 0·0016033 g of sulphur.

Results

Solutions were prepared by dissolving various amounts of sulphur in 100-g portions of a 37·7 per cent. solution of sodium hydroxide at 100° to 110° C. When cool, each solution was weighed and covered with a layer of oil to prevent oxidation. The contents of polysulphide, sulphide, thiosulphate and total sulphur in these solutions were determined by the proposed methods, and, as a check, other methods were used for determining sulphide[6] and thiosulphate[7]; the results are shown in Table I.

I thank Professor Dr. Albin Kurtenacker, former professor of the Technical University of Brünn, for his interest in this work and Miss Klára Lázár for conscientious and precise technical assistance.

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DETERMINATION OF NITROGEN IN ARGON BY GAS CHROMATOGRAPHY

The need for a method of determining nitrogen in argon arose when the latter gas was used as an inert ambient in processing reactive materials, *e.g.*, pure elements for semi-conductors. Nitrogen is one of the major impurities in argon from cylinders, and its presence in amounts greater than normal can indicate the presence of leaks and hence the entry of air into pipes and apparatus. Nitrogen can be separated from argon and oxygen[1] at room temperature by adsorption on a chromatographic column of Linde molecular sieve (type 5A), helium being used as carrier gas. This system is satisfactory when the nitrogen and argon are present in similar proportions. However, if the concentration of nitrogen in the argon is small, say, 50 p.p.m. by volume, then a larger sample must be used. A sample of size sufficient to produce an appreciable response for nitrogen would also produce such a large response for argon that the peak for nitrogen would be hidden and the determination impracticable.

Lard and Horn[1] reported that, at −72° C, oxygen and argon were eluted as separate peaks from Linde molecular sieve 5A, but that nitrogen remained on the column. Tests with mixtures of argon and air showed that this worked well, and nitrogen was not released until the molecular sieve was warmed. The method we adopted was to pass the sample of argon (approximately 50 to 200 ml) through a small trap filled with molecular sieve and cooled in a mixture of acetone and solid carbon dioxide; nitrogen was adsorbed in this trap. Helium was then passed through the trap and fed into a gas-chromatographic column packed with molecular sieve at room temperature, from which it passed to the detector; this produced a large response as the argon and oxygen were purged from the trap. The pen indicating the response returned to the base line when purging was complete, and, at this point, the trap was warmed to release the nitrogen, which produced a peak in the usual way.

A small volume of air was used as a standard source of nitrogen; this air was adsorbed in the sample trap at −72° C and treated in a similar manner to the sample of argon. Nitrogen was removed from the helium carrier gas by passage through a separate trap packed with molecular sieve and cooled in liquid nitrogen.

DETECTOR—

Any detector sensitive to the permanent gases can be used, but an ionisation detector[2] was found to be sufficiently sensitive when used in a manner similar to that described by Willis.[3] In his method, a constant proportion of impurity was introduced into the carrier gas, and the permanent gases then produced a negative response in the detector.

With the helium used as carrier gas in this work, no extra added impurity was needed, as the purification system was not completely effective and sufficient response was obtained. (This technique has been independently developed by Ellis and Forrest.[4]) Sensitivity was improved by polarising the central electrode to +900 volts and by connecting the amplifier to the body of the detector, this being the reverse of the usual procedure. When the equipment was used in this manner, a full-scale deflection of 10 mV on the recorder pen was given by the peak for nitrogen from a 0·05-ml sample of air, *i.e.*, by 40 μl of nitrogen.

As an example of the accuracy attained, a series of nine determinations gave a nitrogen content of 9·8 p.p.m. by volume, with a standard deviation of 16 per cent. A typical recorder diagram is shown in Fig. 1.

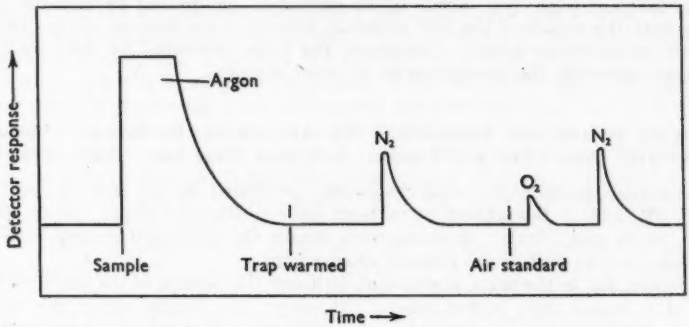

Fig. 1. Typical recorder diagram, with helium flowing at 16 ml per minute

I thank Mr. G. King, Director of Research, Standard Telecommunication Laboratories, for permission to publish this Note.

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STANDARD TELECOMMUNICATION LABORATORIES LTD.
HARLOW, ESSEX

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Received *June 30th*, 1961

Book Reviews

PRINCIPLES AND APPLICATIONS OF PAPER ELECTROPHORESIS. By CH. WUNDERLY. Pp. xii + 253. Amsterdam, London, New York and Princeton: Elsevier Publishing Company; London: D. Van Nostrand Company Ltd. 1961. Price 26s.

This volume is the latest addition to the series of useful Elsevier monographs. As such, it has a certain function to fulfil, *i.e.*, to present the relevant information in a specialised field of science concisely, intelligibly and critically within the limited space allowed. The compression of the absolutely essential facts and references to a small volume is welcomed by the reader as a time-saving simplification of study. He expects a well balanced account of the total subject,

reliable as to facts and figures, thereby relieving him of the burden of further literature searches and checks. In fairness to the author of any monograph, it must be said that the task is not easy and requires long, deep and varied experience in the particular subject. As soon as the scope of the volume is enlarged beyond the subject, the weaknesses of the book become apparent.

Here, one feels that, if Dr. Wunderly had limited himself to writing a monograph on the paper electrophoresis of proteins, the book would have been a complete success. The chapters on proteins, including serum proteins, lipoproteins, glycoproteins, haemoglobins and enzymes, cover the field concisely, lucidly and comprehensively. Particularly useful is the sound advice offered on the quantitative evaluation of protein fractions, about the makes and grades of paper to be used, various staining procedures, photometry, appraisal of reproducibility and sources of systematic errors. The compositions of proteins of various biological fluids, both in man and animal, under normal and pathological conditions are aptly described and excellently illustrated. The less impressive chapters are those on methods and equipment, and the least satisfying ones are those on carbohydrates, amino acids, peptides, organic acids and inorganic ions. One cannot avoid the feeling that here the author is outside his accustomed depth.

The volume is a translation of the original German text, and this is, unfortunately, too obvious on many pages. It becomes irritating when some German technical terms are somewhat clumsily translated so as to rob them of their corresponding meanings in English. Some inconsistencies in the text and inaccuracies, such as in the caption of Fig. 8 (p. 28), quoting the volume of toluene as 50 litres instead of less than 2 litres, attributing the electro-osmotic effect to two different causes on two different pages (pp. 8 and 46) or stating on pp. 15 and 29 that the joulean (*sic!*) heat increases with the square of the field strength, instead of the current, do not tend to inspire the reader with excessive confidence. However, the value presented by the excellent chapters on proteins may outweigh the shortcomings in other respects. D. Gross

Name Reactions in Organic Chemistry. By Alexander R. Surrey. Second Edition. Pp. x + 278. New York and London: Academic Press Inc. 1961. Price $8.00; 64s.

To the hundred-odd selected "name reactions" presented in the first edition (reviewed in *Analyst*, 1954, **79**, 723) a few others have been added—Baeyer-Villiger, Birch, Hunsdiecker, Ivanov, Prins, Stork and Wittig. Investigations during the intervening years have resulted in textual additions to over half of the original monographs.

The biographies are, in the main, unchanged, although the passing of the years has necessitated the addition of a second date in five places. However, the details about the first-named of the Erlenmeyer-Plöchl azlactone synthesis are completely changed, since paternity has been transferred from the father to the son.

This edition is rather more attractively presented than was the first; it has been completely re-set in larger type, and a stouter paper has been used. B. A. Ellis

Reagent Chemicals: American Chemical Society Specifications, 1960. Prepared by the 1960 Committee on Analytical Reagents (W. Stanley Clabaugh, Chairman). Pp. xvi + 564. Washington, D.C.: Applied Publications, American Chemical Society. 1961. Price $10.00.

The two previous editions of "Reagent Chemicals" appeared in 1950 and 1955. Such a publication must keep pace with the changing and ever more exacting needs of the analyst. On the other hand, the periodical revision of such a compilation is no mean task, and the 5-year interval represents a reasonable compromise.

The 1960 edition provides specifications for 233 reagents, an increase of 38 over the number covered in 1955. The additional items include five of the more modern pH indicators and seven organic solvents for use in spectrophotometry.

Flame-photometric procedures have been introduced for determining trace amounts of sodium, potassium, calcium and strontium. Tests for free acid and free alkali by titration have been replaced by pH determinations with a glass electrode, and the Karl Fischer technique is applied more extensively than previously to the determination of water in organic liquids.

On average, the levels of purity demanded by these specifications are closely similar to those of standards used in Britain. The points of divergence, both in limits of individual impurities and in methods of determining them, will interest all producers of high-grade chemicals and those consumers who have special problems. W. C. Johnson

TOOLS OF BIOLOGICAL RESEARCH. Third Series. Edited by HEDLEY J. B. ATKINS, D.M., M.Ch., F.R.C.S. Eng., Hon. F.A.C.S. Pp. xiv + 155. Oxford: Blackwell Scientific Publications. 1961. Price 37s. 6d.

One of the consequences of the rapid proliferation of research techniques that has occurred, particularly in the past two decades, is the difficulty of deciding on the appropriate methods to use in a projected investigation; a decision that requires a wide knowledge of the scope of available techniques and, as importantly, of their inaccuracies and limitations. In the medical sciences, the formation of research teams whose members have received their formal training in widely different disciplines, so that a technique long familiar to one worker may be completely unknown to his colleagues, often adds to the problem of selecting a method that of explaining its principles and practice in a clear and convincing manner. Thus, the biochemist may find himself called upon to expound the facts of chromatography to his medical collaborators or the physicist to instruct his biologically trained fellows in measurement of radioactivity. In such circumstances it is of great value to be able to refer the enquirer to a book or paper in which the facts are clearly and simply set out. This volume, the third of a series of symposia organised by the Surgical Research Society, is just such a book, the techniques described ranging from infra-red and ultra-violet absorption spectroscopy, gas chromatography, the use of ion-exchange resins and of polarography in oxygen measurement to the measurement of radioactivity, the use of ultrasonics in diagnosis and autoradiography.

In each chapter, basic principles underlying the technique are outlined, and the type of information given and limitations inherent in the method are described. The amount of experimental detail given varies from the slight to the extensive, as in the chapter on autoradiography; the former is perhaps the more appropriate in a work not intended as a laboratory manual. The relative values of individual chapters would seem to depend on the ease with which the information they contain can be obtained from other sources; so much has been written at all levels of complexity on the measurement of radioactivity, for example, that it seems unlikely that the small amount of information in this book can be new to anyone. The same is true to some extent of chromatography, with the possible exception of gas chromatography, of which an excellent account is given here. On the other hand, readily accessible accounts of the scope and principles of infra-red and ultra-violet spectroscopy are rather more rare, and these subjects are well treated.

The two chapters on diagnosis by ultrasonics, and the one completely "biological" chapter on chromosome analysis, provide an opportunity for the chemist or physicist to become acquainted with methods more familiar to his medical colleagues, and the introduction by Sir Harold Himsworth could be read with profit by any scientist, whatever his background.

D. W. Moss

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REPRINTS OF "THE DETERMINATION OF NUCLEIC ACIDS IN BIOLOGICAL MATERIALS" BY W. C. HUTCHISON AND H. N. MUNRO

REPRINTS of the Review Paper "The Determination of Nucleic Acids in Biological Materials" by W. C. Hutchison and H. N. Munro, published in this issue of *The Analyst* (pp. 768–813), will be available shortly from the Assistant Secretary, the Society for Analytical Chemistry, 14 Belgrave Square, London, S.W.1, at 5s. per copy, post free. A remittance for the correct amount, made out to The Society for Analytical Chemistry, MUST accompany the order; these reprints are not obtainable through Trade Agents.

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